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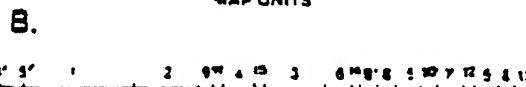
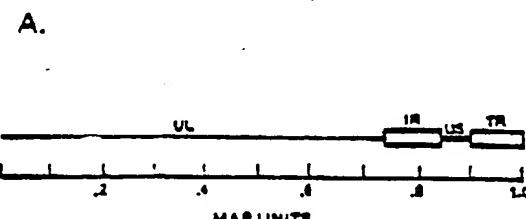
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(54) Attenuated herpesviruses, herpesviruses which include foreign dna encoding an amino  
acid sequence and vaccine containing same

(57) A recombinant herpesvirus of turkeys comprising  
a foreign DNA inserted into a non-essential region of  
the herpesvirus of turkeys viral genome, which is capable  
of being expressed in a host cell infected with the  
herpesvirus of turkeys.

Figure 1



## Description

BACKGROUND OF THE INVENTION

5 Within this application several publications are referenced by Arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entirety are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

10 The advent of recombinant DNA techniques has made it possible to manipulate the naturally occurring DNA sequences within an organism (the genome) in order to change in some manner the functions of the organism through genetic engineering. The present invention concerns organisms defined as viruses that infect animals and contain DNA as their genetic material; specifically viruses belonging to the herpesvirus group (herpesviruses) (23). This group of viruses comprise a number of pathogenic agents that infect and cause disease in a number of target species: swine, cattle, chickens, horses, dogs, cats, etc. Each herpesvirus is specific for its host species, but they are all related in the 15 structure of their genomes, their mode of replication, and to some extent in the pathology they cause in the host animal and in the mechanism of the host immune response to the virus infection.

15 The types of genetic engineering that have been performed on these herpesviruses consist of cloning parts of the virus DNA into plasmids in bacteria, reconstructing the virus DNA while in the cloned state so that the DNA contains deletions of certain sequences, and furthermore adding foreign DNA sequences either in place of the deletions or at sites removed from the deletions. The usual method is to make insertions of the foreign DNA into the viral sequences, although the foreign DNA could be attached to the end of the viral DNA as well. One utility of the addition of foreign sequences is achieved when the foreign sequence encodes a foreign protein that is expressed during viral infection of the animal. A virus with these characteristics is referred to as a vector, because it becomes a living vector which will carry and express the foreign protein in the animal. In effect it becomes an elaborate delivery system for the foreign protein.

25 The prior art for this invention stems first from the ability to clone and analyze DNA while in the bacterial plasmids. The techniques that are available for the most part are detailed in Maniatis et al. (1). This publication gives state-of-the-art general recombinant DNA techniques.

30 The application of recombinant DNA techniques to animal viruses has a relatively recent history from about 1980. The first viruses to be engineered have been the smallest ones - the papovaviruses. These viruses contain 3000-4000 base pairs (bp) of DNA in their genome. Their small size makes analysis of their genomes relatively easy and in fact most of the ones studied (SV40, polyoma, bovine papilloma) have been entirely sequenced. Because these virus particles are small and cannot accommodate much extra DNA, and because their DNA is tightly packed with essential sequences (that is, sequences required for replication), it has not been possible to engineer these viruses as live 35 vectors for foreign gene expression. Their entire use in genetic engineering has been as defective replicons for the expression of foreign genes in animal cells in culture (roughly analogous to plasmids in bacterial systems) or to their use in mixed populations of virions in which wild type virus acts as a helper for the virus that has replaced an essential piece of DNA with a foreign gene. The studies on papovaviruses do not suggest or teach the concept of living virus vectors as delivery systems for host animals.

40 The next largest DNA animal viruses are the adenoviruses. In these viruses there is a small amount of nonessential DNA that can be replaced by foreign sequences. The only foreign genes that seem to have been expressed in adenoviruses are the T-antigen genes from papovaviruses (2,3,4,5), and the herpes simplex virus thymidine kinase gene (28). It is possible, given this initial success, to envision the insertion of other small foreign genes into adenoviruses. However 45 the techniques used in adenoviruses do not teach how to obtain the same result with herpesviruses. In particular, these results do not identify the nonessential regions in herpesviruses wherein foreign DNA can be inserted, nor do they teach how to achieve the expression of the foreign genes in herpesviruses, e.g. which promoter signals and termination signals to use.

45 Another group of animal viruses that have been engineered are the poxviruses. One member of this group, vaccinia, has been the subject of much research on foreign gene expression. Poxviruses are large DNA-containing viruses 50 that replicate in the cytoplasm of infected cells. They have a structure that is very unique among viruses - they do not contain any capsid that is based upon icosahedral symmetry or helical symmetry. In theorizing on the origin of viruses, the poxviruses are the most likely ones to have originated from bacterial-like microorganisms through the loss of function and degeneration. In part due to this uniqueness, the advances made in the genetic engineering of poxviruses cannot be directly extrapolated to other viral systems, including herpesviruses. Vaccinia recombinant virus constructs have 55 been made in a number of laboratories that express the following inserted foreign genes: herpes simplex virus thymidine kinase gene (6,7), hepatitis B surface antigen (8,9,29), herpes simplex virus glycoprotein D gene (8,29), influenza hemagglutinin gene (10, 11), malaria antigen gene (12), and vesicular stomatitis glycoprotein G gene (13). The general overall features of the vaccinia recombinant DNA work are similar to the techniques used for all the viruses, especially as they relate to the techniques in reference (1). However in detail, the vaccinia techniques do not teach how to engi-

neer herpesviruses. Vaccinia DNA is not infectious, so the incorporation of foreign DNA must involve an infection/transfection step that is not appropriate to other viruses, and vaccinia has unique stability characteristics that make screening easier. In addition, the signal sequence used by promoters in vaccinia are unique and will not work in other viruses. The utility of vaccinia as a vaccine vector is in question because of its close relationship to human smallpox and its known pathogenicity to humans. The use of host-specific herpesviruses promises to be a better solution to animal vaccination.

5 Herpesviruses contain 100,000 to 150,000 base pairs of DNA as their genetic material, and several areas of the genome have been identified that are dispensable for the replication of virus *in vitro* in cell culture. Modifications of these regions of the DNA are known to lower the pathogenicity of the virus, i.e. to attenuate the virus, for an animal species.  
10 For example, inactivation of the thymidine kinase gene renders human herpes simplex virus non-pathogenic (45), and pseudorabies virus of swine non-pathogenic (46 and 47).

15 Removal of part of the repeat region renders human herpes simplex virus non-pathogenic (48 and 49). A repeat region has been identified in Marek's disease virus that is associated with viral oncogenicity (50). A region in herpesvirus saimiri has similarly been correlated with oncogenicity (51). However, modifications in these repeat regions do not teach the construction of attenuated pseudorabies viruses with deletions in repeat sequences.

20 The degree of attenuation of a virus is important in the utility of the virus as a vaccine. Deletions which cause too much attenuation of the virus will result in a vaccine that fails to elicit an adequate immune response.

25 The herpesviruses are known to cause a variety of latent and recurrent infections in human and other vertebrates and are often known to infect a fungus and an oyster. Among the conditions associated with herpesvirus infections are fever blisters caused by herpes simplex type 1, genital herpes causes by herpes simplex type 2, and chickenpox in children and shingles in adults cause by herpes zoster infection. Pseudorabies virus (PRV), a Class D herpesvirus, induces Aujesky's disease, an acute and often fatal nervous condition, in domestic and wild animals.

30 Among the herpesviruses, only herpes simplex of humans and, to a limited extent, herpes saimiri of monkeys have been engineered to contain foreign DNA sequences previous to this disclosure. The earliest work on the genetic manipulation of herpes simplex virus involved the rescue of temperature sensitive mutants of the virus using purified restriction fragments of DNA (14). This work did not involve cloning of the DNA fragments into the viral genome. The first use of recombinant DNA to manipulate herpes simplex virus involved cloning a piece of DNA from the L-S junction region into the unique long region of the DNA, specifically into the thymidine kinase gene (15). This insert was not a foreign piece of DNA, rather it was a naturally occurring piece of herpesvirus DNA that was duplicated at another place in the genome. This piece of DNA was not engineered to specifically express any protein, and thus it did not teach how to express protein in herpesviruses. The manipulation of herpes simplex next involved the creation of deletions in the virus genome by a combination of recombinant DNA and thymidine kinase selection. The first step was to make a specific deletion of the thymidine kinase gene (16). The next step involved the insertion of the thymidine kinase gene into the genome at a specific site, and then the thymidine kinase gene and the flanking DNA at the new site were deleted by a selection against thymidine kinase (17). In this manner herpes simplex alpha-22 gene has been deleted (17). In the most recent refinement of this technique, a 15,000 bp sequence of DNA has been deleted from the internal repeat of herpes simplex virus (18).

35 The insertion of genes that encode protein into primate herpesviruses have involved seven cases: the insertion of herpes simplex glycoprotein C back into a naturally occurring deletion mutant of this gene in herpes simplex virus (19); the insertion of glycoprotein D of herpes simplex type 2 into herpes simplex type 1 (20), again with no manipulation of promoters since the gene is not really 'foreign'; the insertion of hepatitis B surface antigen into herpes simplex virus under the control of the herpes simplex ICP4 promoter (21); and the insertion of bovine growth hormone into herpes saimiri virus with an SV40 promoter that in fact didn't work in that system (an endogenous upstream promoter served to transcribe the gene) (22). Two additional cases of foreign genes (chicken ovalbumin gene and Epstein-Barr virus nuclear antigen) have been inserted into herpes simplex virus (30), and glycoprotein X of pseudorabies virus has been inserted into herpes simplex virus (31).

40 These limited cases of deletion and insertion of genes into herpesviruses demonstrate that it is possible to genetically engineer herpesvirus genomes by recombinant DNA techniques. The methods that have been used to insert genes involve homologous recombination between the viral DNA cloned on plasmids and purified viral DNA transfected into the same animal cell. In aggregate this is referred to as the homologous recombination technique. This technique with minor modifications has been adaptable to other herpesviruses that we have engineered. However, the extent to which one can generalize the location of the deletion and the sites for insertion of foreign genes is not obvious from these previous studies. Furthermore, it is also not obvious that non-primate herpesviruses are amenable to the same techniques as the primate herpesviruses, and that one could establish a targeted approach to the deletion, insertion, and expression of foreign genes.

45 Infectious bovine rhinotracheitis (IBR) virus, an alpha-herpesvirus with a class D genome, is an important pathogen of cattle. It has been associated with respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal diseases (37). Cattle are the normal hosts of IBR virus, however it also infects goats, swine, water buffalo, wildebeest, mink and ferrets. Experimental infections have been established in muledeer, goats, swine, ferrets and rabbits

(38).

Conventional modified live virus vaccines have been widely used to control diseases caused by IBR. These vaccine viruses may revert to virulence, however. More recently, killed virus IBR vaccines have been used, but their efficacy appears to be marginal.

5 IBR has been analyzed at the molecular level as reviewed in (39). A restriction map of the genome is available in this reference, which will aid in the genetic engineering of IBR according to the methods provided by the present invention. No evidence has been presented that IBR has been engineered to contain a deletion or an insertion of foreign DNA.

10 Marek's disease virus (MDV) causes foul paralysis, a common lymphoproliferative disease of chickens. The disease occurs most commonly in young chickens between 2 and 5 months of age. The prominent clinical signs are progressive paralysis of one or more of the extremities, incoordination due to paralysis of legs, drooping of the limb due to wing involvement, and a lowered head position due to involvement of the neck muscles. In acute cases, severe depression may result. In the case of highly oncogenic strains, there is characteristic bursal and thymic atrophy. In addition, there are lymphoid tumors affecting the gonads, lungs, liver, spleen, kidney and thymus (37).

15 All chicks are vaccinated against MDV at one day of age to protect the chick against MDV for its lifetime. One vaccine method for MDV involves using turkey herpesvirus (HVT). It would be advantageous to incorporate other antigens into this vaccination at one day of age, but efforts to combine vaccines have not proven satisfactory to date due to competition and immunosuppression between pathogens. The multivalent vaccines engineered in this invention are a novel way to simultaneously vaccinate against a number of different pathogens.

20 A restriction map of both MDV (43) and HVT (34) are available in the literature. There is no evidence to suggest that anyone has successfully created a deletion or insertion of foreign DNA into MDV or HVT prior to this disclosure.

Other herpesviruses contemplated to be amenable to these procedures are feline herpesvirus (FHV), equine herpesvirus (EHV), and canine herpesvirus (CHV). These pathogens cause disease in each of their respective hosts. Feline herpesvirus causes feline rhinotracheitis, an acute upper respiratory tract infection characterized by fever, pronounced sneezing, nasal and lacrimal secretions, and depression. The virus may cause corneal ulceration and abortion. The nasal passages and turbinates show focal necrosis, and the tonsils are enlarged and hemorrhagic. Equine herpesvirus causes rhinopneumonitis, abortion, exanthema of the genitals and occasionally neurologic disease. The acute disease is characterized by fever, anorexia and a profuse, serous nasal discharge. The neurologic symptoms, when present, consist of ataxia, weakness and paralysis. Canine herpesvirus causes severe illness in young puppies, where mortality may reach 80%. The disease is characterized by viremia, anorexia, respiratory illness, abdominal pain, vomiting and incessant crying. Generally, there is no fever. The principal lesions are disseminated necrosis and hemorrhages in the kidneys, liver and lungs.

35 The molecular biology of the feline, equine and canine herpesviruses are in their initial stages. Partial restriction maps are available for equine herpesvirus, and in progress in at least one lab for the feline herpesvirus. Beyond this type of genome analysis, no evidence for the deletion or insertion of foreign genes into these viruses is available.

The present invention involves the use of genetically engineered herpesvirus to protect animals against disease. It is not obvious which deletions in herpesviruses would serve to attenuate the virus to the proper degree. Even testing vaccine candidates in animal models, e.g. mice, does not serve as a valid predictor of the safety and efficacy of the vaccine in the target animal species, e.g. swine.

40 Another subject of the present invention is a vaccine for pseudorabies virus (herpesvirus suis, suid herpesvirus 1, or Aujesky's disease virus) disease of swine. Swine are the natural host of pseudorabies virus in which infection in older animals is commonly inapparent but may be characterized by fever, convulsions, and death particularly in younger animals. Pseudorabies also infects cattle, sheep, dogs, cats, ferrets, foxes, and rats (37) where the infection usually results in death. Death is usually preceded by intense pruritus, mania, encephalitis, paralysis, and coma. Traditional live vaccines are available for use in swine, but they are lethal for the other animals. An improved vaccine for pseudorabies would induce a more reliable immune response in swine, would be specifically attenuated to be incapable of reversion to virulence, and would not cause disease in other hosts.

50 Pseudorabies virus, an alpha-herpesvirus of swine, has a genome of class D (23); that is it contains two copies of a single repeat region, one located between the unique long and unique short DNA region and one at the terminus of the unique short region (see Figure 1). Herpes simplex virus is an alpha-herpesvirus with a class E genome (23); that is it contains two copies of each of two repeats. Herpes saimiri is a gamma-herpesvirus with a class B genome; that is, it contains numerous reiterations of the same sequence at both termini (23). As the genome structure differs significantly between these different classes of herpesviruses, and because the different viruses attack different cells within their hosts and elicit different pathologies, it is necessary in each instance to establish which specific regions can be removed in order to attenuate and which regions can be altered to express foreign genes.

55 Pseudorabies virus has been studied using the tools of molecular biology including the use of recombinant DNA techniques. BamHI, KpnI, and BglII restriction maps of the virus genome have been published (24, 27). DNA transfection procedures have been utilized to rescue temperature sensitive and deletion mutants of the virus by the homologous recombination procedure (24). There are two examples of deletions that have been made in the pseudorabies virus

genome - one is a thymidine kinase gene deletion (25), also disclosed in Patent No. 4,514,497 entitled "Modified Live Pseudorabies Virus-es". This patent teaches thymidine kinase deletions only and does not suggest other attenuating deletions, nor does it suggest insertion of foreign DNA sequences. The other reference involves the deletion of a small DNA sequence around a HindIII restriction site in the repeat region (26) upon which European Patent Publication No. 0141458, published on May 15, 1985, corresponding to European Patent Application No. 84201474.8, filed on October 12, 1984 is based. This patent application does not teach or suggest attenuating deletions nor does it teach or suggest the insertion of DNA sequences into pseudorabies virus.

The present invention concerns deletions which have been introduced into the pseudorabies virus genome at sites previously undisclosed. Foreign DNA sequences have also been introduced into the attenuated pseudorabies virus genome and expressed as proteins. One embodiment of the invention concerns a vaccine useful for preventing pseudorabies and other swine diseases with a single inoculum.

Other relevant pseudorabies literature disclosed herein, concerns the presence of naturally-occurring deletions in the genome of two vaccine strains of pseudorabies viruses (27). These deletions are responsible, at least in part, for the attenuated nature of these vaccines however they do not occur in a repeat sequence and do not suggest the attenuation of pseudorabies virus by deleting a portion of a repeat sequence. Such naturally-occurring deletions do not teach methods for making these deletions starting with wild type pseudorabies virus DNA, nor do they suggest other locations at which to make attenuating deletions. There are no examples of naturally-occurring insertions of foreign DNA in herpes viruses.

The natural host of pseudorabies virus is swine, in which infection is commonly inapparent but may be characterized by convulsions and paralysis. Pseudorabies virus also infects cattle, sheep, dogs, cats, foxes and mink, where infection usually results in death of the host. The predominant visible feature of pseudorabies viral infection is extreme pruritis generally resulting in host mutilation of the involved area. Violent excitement, fits and paralysis, all symptoms of encephalomyelitis, precede death which usually occurs within a few days following onset of clinical signs.

Pseudorabies virus disease in swine is a serious concern to governmental bodies worldwide. In the United States, swine from infected herds cannot be sold except to slaughterhouses. Several individual states have separately enacted eradication control practices against pseudorabies. At the current time, any animal vaccinated for pseudorabies disease is treated as though it were infected with pseudorabies virus and is subject to the same regulatory constraints. This is due primarily to the lack of a diagnostic test to differentiate vaccinated from infected animals.

The research and development trend among traditional vaccine manufacturers has generally emphasized research leading to vaccines that are based upon virus subunits rather than live viruses. This departure from live virus vaccines is due partly to the recognized safety aspect of subunit vaccines, and their unlikelihood of containing infectious live viruses. Another reason for developing a subunit vaccine has been to allow for the development of a diagnostic test that would accompany the vaccine and would differentiate vaccinated from infected animals, thereby escaping from the regulatory burden following use of other vaccines.

Subunit vaccines also have limitations. They contain a limited number of viral antigens compared to those produced by live viruses. This paucity of antigens produces a weak immune response of short duration in the vaccinated animal at considerably greater cost than a live virus vaccination. However, the limited spectrum of antigens in the subunit vaccine allows the vaccinated swine to be distinguished from swine which have been infected with the wild-type virus. The ability to distinguish vaccinated from infected swine is a crucial property of a pseudorabies vaccine because none of the known vaccines prevent the vaccinated animals from being super-infected by the wild-type virus. While the vaccinated animals do not become sick upon super-infection, there is strong evidence that they may become carriers of the wild-type virus and pass the wild-type virus to other swine.

In any eradication program aimed at eliminating pseudorabies virus, a vaccine provided with characteristics which would allow vaccinated animals to be distinguished from animals infected with wild-type virus would be advantageous. The subunit vaccines have high cost and poor efficacy but an animal vaccinated with this type of vaccine will produce antibodies only to the limited spectrum of antigens present in the vaccine. By sampling the serum of the swine, it is possible to show that the vaccinated animal has antibodies only to the antigens contained in the vaccine while an animal infected with the wild-type virus would have antibodies against a wider range of antigens. A subunit vaccine used in this way to differentiate vaccinated from pseudorabies infected animals has been disclosed in European Patent Application No. 8540074.4, filed on September 4, 1985, published November 27, 1985 as European Publication No. 0162738 and entitled "Production of Pseudorabies Virus Subunit Vaccines". This published patent application does not teach or suggest the construction or use of a similar diagnostic test in conjunction with a live virus vaccine. The vaccination of an animal with a live virus which would result in an immune response distinguishable from wild-type infection would also have the further advantages of low cost and high efficacy associated with live virus vaccines.

Deletions in genes coding for viral antigens have been described previously. A spontaneous deletion in the glycoprotein C gene of herpes simplex virus (52), a spontaneous deletion in the glycoprotein A gene of Marek's disease virus (53) a spontaneous deletion in the glycoprotein A gene (also called glycoprotein g1) of PRV (27,55) and the absence or greatly reduced amount of glycoprotein gIII in some PRV mutants (54) are known. However, all of these deletions arose spontaneously in an uncontrolled process. Hence, it has not been possible to direct deletions to DNA encoding for spe-

cific antigens to control the deletion process and direct the deletions to antigens particularly suitable as diagnostic markers.

The presence or absence of particular antigens in any infectious disease can be exploited as a diagnostic test for the infectious disease agent. This presence or absence forms the basis for all immunological diagnostic tests, which differ only in the details of their specific immunological approach. Publications pertinent to the current invention include Wathan and Wathan (54) who reported that either the gI gene or the gIII gene could be deleted from PRV and suggested that the resulting virus could be used for distinguishing vaccinated from infected swine. However, they did not describe the methodology necessary to create the vaccine, they did not demonstrate the utility of such a vaccine in serological tests and they did not in any other way prove the feasibility of such a vaccine.

Van Oirschot, et al. (56), have used a special monoclonal-based immunological detection system for gI of PRV and have shown that pigs inoculated with naturally-occurring vaccine strains which are missing at least a portion of the gI gene can be differentiated from pigs infected by wild-type PRV. However, this diagnostic test may be used for any of several vaccines against PRV that are already existing in both Europe and the U.S. without differentiating which vaccine was used. This limits the usefulness of this diagnostic, since the vaccines which are detectable have differing biological and virulence properties.

The approach of deleting a gene to attenuate a virus coupled with a diagnostic for that gene, provides a vaccine that can be differentiated from any of the currently used PRV vaccines and from wild-type PRV. It is important to be able to differentiate a new, safer vaccine from those currently used because pigs receiving the current vaccines are all regulated during eradication programs to the same extent as those infected with wild-type PRV.

Antigens of choice for the purpose of a diagnostic marker would have the following characteristics: 1) the antigens and their genes would be non-essential for the production of infectious virus in tissue culture; and 2) the antigen would elicit a major serological response in the animal, but is preferably not an important neutralizing antigen.

The present invention therefore involves the ability to attenuate pseudorabies virus of swine to create a live virus vaccine and the ability to distinguish whether an animal has been given the vaccination or whether the animal has been infected by wild-type pseudorabies virus.

#### SUMMARY OF THE INVENTION

The present invention provides a hybrid, nonprimate herpesvirus comprising DNA which includes a sequence essential for viral replication of the hybrid, non-primate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate herpesvirus and at least one foreign DNA sequence.

Also provided is an attenuated, nonprimate herpesvirus comprising DNA which includes a sequence essential for viral replication of the attenuated, nonprimate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate herpesvirus, from which at least a portion of a repeat sequence has been deleted.

The present invention further provides an attenuated, hybrid, nonprimate herpesvirus. This virus comprises DNA which includes a sequence essential for viral replication of the attenuated, hybrid, nonprimate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate herpesvirus and at least one foreign DNA sequence.

Attenuated pseudorabies viruses are also provided which are comprised of DNA including a sequence essential for replication of the attenuated virus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring pseudorabies virus, and at least one foreign DNA sequence adapted for expression in a host and encoding an amino acid sequence which is antigenic in the host.

Vaccines comprised of an effective immunizing amount of a virus of this invention and a suitable carrier are useful for immunizing animals against pseudorabies virus disease. Multivalent vaccines, also comprised of an immunizing amount of a virus of this invention and a suitable carrier are useful for immunizing animals against pseudorabies virus disease and at least one other pathogen.

The invention additionally provides methods of preparing attenuated pseudorabies viruses including at least one foreign DNA sequence and methods of immunizing animals with vaccines comprised of these viruses.

The invention further provides an isolated nucleic acid molecule which encodes swine rotavirus glycoprotein 38 and an isolated nucleic acid molecule which encodes swine parvovirus B capsid protein.

Also provided are attenuated pseudorabies virus comprising DNA which includes a sequence essential for replication of the attenuated pseudorabies virus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring pseudorabies virus. This DNA encodes, in an animal infected with the attenuated pseudorabies virus, mRNAs which are translated into antigenic pseudorabies virus gene products which invoke in the infected animal an immunological response distinguishable from an immunological response invoked in an animal infected with a naturally-occurring pseudorabies virus.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 Details of Wild Type Iowa S-62 A Strain

5 A. Diagram of PRV genomic DNA showing the unique long region (UL), the unique short region (US), the internal repeat region (IR), and the terminal repeat region (TR).

B. BamHI restriction enzyme map of PRV. Fragments are numbered in order of decreasing size.

10 FIG. 2 Details of S-PRV-004 Construction and Map Data

A. Detailed map of BamHI #8' and #8. The location of the internal repeat (IR) region is shown.

B. Detailed map of BamHI #8'-TK-8 fragment ultimately present in the recombinant virus.

15 C. Diagram of the S-PRV-004 DNA genome showing the location of the HSV-1 TK gene inserted into the junction region between the UL and IR regions.  
Restriction Enzyme Legend: B = BamHI; K = KpNI; N = Ndel; P = Pvull; S = StuI.

20 FIG. 3 Details of S-PRV-005 Construction and Map Data

A. Detailed map of BamHI #5. The HSV-1 TK gene fused to the HSV-1 ICP4 promoter is shown on a Pvull fragment.

25 B. Detailed map of BamHI #5 after the insertion of the TK gene construct.

C. Diagram of the S-PRV-005 DNA genome showing the location of the TK gene inserted into both copies of BamHI #5 in the repeat region of the genome and the creation of new deletions.  
Restriction Enzyme Legend: B = BamHI; H = HindIII; Hp = HpaI; K = Kpnl; P = Pvull; X = XbaI.

30 FIG. 4 Construction of the Foreign DNA Insert Used in S-PRV-010

A. Diagram of the relevant portion of pJF751 that contains the lac Z (beta-galactosidase) gene. The position of the TAA termination codon for the polypeptide is indicated.

35 B. Diagram of the promoter sequence from the HSV-1 TK gene.

C. Diagram of the RsaI fragment of the TK gene now with BamHI modified ends.

40 D. Diagram of the final plasmid that contained the lac Z gene fused to the HSV-1 TK promoter.  
Restriction Enzyme legend: B = BamHI; Ba = Ball; Bc = BclI; Bg = BgIII; H = HindIII; Ha = HaeIII; N = Ndel; R = RsaI; X = XbaI.

45 FIG. 5 Details of S-PRV-010 Construction and Map Data

A. Detailed map of BamHI #5. The lac Z gene (beta-galactosidase) fused to the HSV-1 TK promoter is shown on an XbaI fragment (see Fig. 4): The position of the deletion in S-PRV-002 is shown.

B. Detailed map of BamHI #5 after the insertion of the lac Z gene construct.

50 C. Diagram of the S-PRV-010 genome DNA showing the location of the lac Z gene into both copies of BamHI #5 in the repeat region of the genome.  
Restriction Enzyme Legend: B = BamHI; Bc = BclI; H = HindIII; Hp = HpaI; K = Kpnl; N = Ndel; X = XbaI.

55 FIG. 6 Details of S-PRV-007 Construction and Map Data

A. Detailed map of BamHI #5 from S-PRV-005.

EP 0 794 257 A1

B. Detailed map of BamHI #5 after the substitution of the TK gene with the swine rotavirus gp38 gene.

C. Diagram of the S-PRV-007 DNA genome showing the location of the gp38 gene inserted into both copies of BamHI #5 in the repeat regions of the genome.

Restriction Enzyme Legend: B = BamHI; H = HindIII; K = KpnI.

FIG 7 Construction of the Foreign DNA Insert Used in S-PRV-007.

FIG 8 Details of S-PRV-012 Construction and Map Data.

A. Detailed map of PRV extending from BamHI #10 through BamHI #7.

B. Detailed map of PRV extending from BamHI #10 through BamHI #7 after the insertion of the TK gene into the recombinant virus.

C. Diagram of the S-PRV-012 DNA genome showing the location of the TK gene inserted into the gpX region and the creation of a deletion that removes most of the coding region of the gpX gene and renders the virus unable to synthesize the gpX polypeptide.

Restriction Enzyme Legend: B = BamHI; K = KpnI; N = Ndel; P = Pvull; Ps = PstI; S = StuI.

FIG 9 Details of S-PRV-013, S-PRV-014, and S-PRV-016 Construction and Map Data.

A. Detailed map of PRV extending from BamHI #10 through BamHI #7.

B. Detailed map of PRV extending from BamHI #10 through BamHI #7 after the insertion of the lac Z gene into the recombinant virus.

C. Diagram of the S-PRV-013 DNA genome showing the location of the lac Z gene inserted into the gpX region and the creation of a deletion that removed most of the coding region of the gpX gene and rendered the virus unable to synthesize the gpX polypeptide. Other deletions in the TK region and repeat regions are shown by (▲).

D. Diagram of the S-PRV-014 DNA genome showing the location of the lac Z gene inserted into the gpX region and the creation of a deletion that removed most of the coding region of the gpX gene and rendered the virus unable to synthesize the gpX polypeptide. There are no other deletions in this virus.

E. Diagram of the S-PRV-016 DNA genome showing the location of the lac Z gene inserted into the gpX region and the creation of a deletion that removed most of the coding region of the gpX gene and rendered the virus unable to synthesize the gpX polypeptide. Other deletions in the repeat regions are shown by (▲).

Restriction Enzyme Legend: B = BamHI; Ba = Ball; K = KpnI; N = Ndel; Ps = PstI; S = StuI.

FIG 10A and 10B Swine rotavirus gp38 Gene Sequence in pSY565.

FIG 11A and 11B Swine parvovirus B gene sequence in pSY875

FIG. 12 Swine parvovirus B gene construction with signal sequence

A. pSY864 which contains the B gene from Accl at nucleotide #391 to Rsal site at nucleotide #2051 cloned between the BamHI site in BamHI #10 and the Ndel site in BamHI #7.

B. pSY957 which contains the Sall fragment from pSY864 cloned into a polylinker in pSP65 so that XbaI sites flank the insert.

Legend. pSP = E. coli plasmid; PRV = pseudorabies virus DNA; PPV = porcine parvovirus DNA; Pv = Pvull; RV = EcoRV; Ps = PstI; B = BamHI; A = Accl; R = Rsal; N = Ndel; Sa = Sall; Sm = SmaI; S = StuI; X = XbaI; gpX pro = glycoprotein X promoter; gpX pA = glycoprotein X polyadenylation signal sequences.

FIG. 13

Details of S-PRV-020 Construction and Map Data

5 A. Detailed map of PRV extending from BamHI #10 through BamHI #7 showing the parvovirus B gene that will replace the gpX gene.

10 B. Detailed map of PRV from BamHI #10 through BamHI #7 after the insertion of the swine parvovirus B gene in place of the gpX gene.

15 C. Diagram of the S-PRV-020 genome showing the location of the swine parvovirus B gene inserted into the gpX region of PRV.

20 Restriction Enzyme Legend: B=BamHI; Ps=PstI; Sa=Sall; N=Ndel; S=StuI; A=AccI; R=RsaI.

FIG. 14

Details of S-PRV-025 construction and map data

25 A. Region of S-PRV-002 starting virus showing BamHI #5 fragment. The parvovirus B gene XbaI fragment from pSY957 is diagrammed below showing how it will be inserted into the XbaI site by direct ligation.

30 B. Region of BamHI #5 after insertion of the parvovirus B gene.

35 C. Location of the parvovirus B gene inserted into both copies of the repeat in S-PRV-025.

Legend:

40 B = BamHI; H = HindIII; X = XbaI; S = Sall; pA = glycoprotein X polyadenylation signal sequences; UL = unique long region; US = unique short region; IR = internal repeat region; TR = terminal repeat region.

FIG. 15

Details of S-PRV-029 Construction and Map Data

45 A. Detailed map of PRV extending from BamHI #10 through BamHI #7 showing the lac Z gene that will replace the gpX gene.

50 B. Detailed map of PRV extending from BamHI #8' through BamHI #8 at the junction of the unique long region and the internal repeat region (IR). The lac Z gene as a Sall fragment will replace the DNA between the StuI sites bracketing the junction.

55 C. Diagram of the S-PRV-029 genome showing the locations of the lac Z genes in the gpX region and the junction region.

60 Restriction Enzyme Legend: B=BamHI; Ps=PstI; Sa=Sall; N=Ndel; S=StuI; Ba=BalI; K=KpnI.

FIG. 16

Restriction Map of Deleted S-IBR-002 EcoRI B Fragment and EcoRI F Fragment

65 An 800 bp deletion including EcoRV and BgIII restriction sites was mapped in both repeat fragments.

FIG. 17

Construction of Recombinant S-IBR-004 Virus

70 S-IBR-004 is an IBR recombinant virus carrying an inserted foreign gene (NEO) under the control of the PRV gpX promoter. A new XbaI site was created at the small unique region and the original SacI site was deleted.

FIG. 18

Construction of Recombinant S-IBR-008 Virus

75 S-IBR-008 is a recombinant IBR virus that has a bovine rota glycoprotein gene and the plasmid vector inserted in the XbaI site on the unique long region. A site specific deletion was created at the [SacI] site due to the loss of NEO gene in the small unique region.

FIG. 19

Details of HVT Construction and Map Data

80 A. BamHI restriction fragment map of HVT. Fragments are numbered in order of decreasing size; letters refer to small fragments whose comparative size has not been determined.

85 B. BamHI #16 fragment showing location of betagalactosidase gene insertion in S-HVT-001.

C. BamHI #19 fragment showing location of betagalactosidase gene insertion.

Legend: B = BamHI; X = Xhol; H = HindIII; P = PstI; S = Sall; N = NdeI; R = EcoRI.

5 FIG. 20. Western blot of proteins released into the medium of PRV infected cells, showing the absence of gpX in S-PRV-012 and S-PRV-013 but its presence in wild-type PRV-000. Lanes: (A) molecular weight markers, (B) uninfected Vero cells, (C) wild-type PRV, (D) S-PRV-012, (E) S-PRV-013.

10 FIG. 21. Diagnostic test for the presence of antibodies against gpX in the serum of a pig vaccinated with S-PRV-013 on Day 0 and challenged with wild-type pseudorabies virus on Day 28.

#### DETAILED DESCRIPTION OF THE INVENTION

15 The present invention provides a hybrid, nonprimate herpesvirus comprising DNA which includes a sequence essential for viral replication of the hybrid, non-primate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate herpesvirus and at least one foreign DNA Sequence. The sequence essential for viral replication of the hybrid, nonprimate herpesvirus may be synthetic or derived from a naturally-occurring nonprimate herpesvirus.

20 The foreign DNA sequence may be adapted for expression in a host and encode an amino acid sequence. In one embodiment of the invention, the foreign DNA sequence is adapted for expression by a herpesvirus promoter. The herpesvirus promoter may be an endogenous upstream herpesvirus promoter or an inserted upstream herpesvirus promoter. Examples of such herpesvirus promoters include, but are not limited to, the herpes simplex type ICP4 protein promoter, the herpes simplex type I thymidine kinase promoter, the pseudorabies thymidine kinase promoter, the pseudorabies immediate early gene promoter, the pseudorabies glycoprotein X promoter or the pseudorabies glycoprotein 25 92 promoter.

25 The amino acid sequence encoded by the foreign DNA sequence may be a polypeptide. Furthermore, the polypeptide may be a protein. In one embodiment of the invention, the protein, when expressed in the host, is antigenic. In a further embodiment of the invention, the protein is swine rotavirus glycoprotein 38. In yet another embodiment of the invention, the protein is bovine rotavirus glycoprotein 38. In yet a further embodiment of the invention, the protein is swine parvovirus B capsid protein.

30 The hybrid, nonprimate herpesvirus may comprise DNA of which at least a portion is present in a sequence essential for replication of a naturally-occurring alpha-herpesvirus. The alpha-herpesvirus may be a pseudorabies virus, infectious bovine rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I. Additionally the alpha-herpesvirus may be a class D herpesvirus. The class D herpesvirus may be pseudorabies virus, infectious bovine 35 rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I. In one embodiment of the invention, the alpha-herpesvirus is an infectious bovine rhinotracheitis virus and the foreign DNA encodes the *Escherichia coli* neomycin resistance gene. This foreign DNA sequence may also be under the control of an inserted pseudorabies virus glycoprotein X promoter. Such a virus has been constructed, designated S-IBR-004, and deposited with the Ammerican Type Culture Collection (ATCC), Rockville, Maryland, under Accession No. VR 2134.

40 In another embodiment of the invention, the alpha-herpesvirus is an infectious bovine rhinotracheitis virus with a deletion in the unique short sequence. Furthermore, the foreign DNA sequence may encode the bovine rotavirus glycoprotein 38 gene. This virus, designated S-IBR-008, has been constructed and deposited with the ATCC under Accession No. VR 2141.

45 Additionally the hybrid, nonprimate herpesvirus may comprise DNA of which at least a portion is present in a sequence essential for replication of a naturally-occurring gamma-herpesvirus. The gamma-herpesvirus may be Marek's disease virus or herpesvirus of turkeys. Moreover the gamma-herpesvirus may be a class E herpesvirus. The class E herpesvirus may be Marek's disease virus or herpesvirus of turkeys.

50 Also provided is an attenuated, nonprimate herpesvirus comprising DNA which includes a sequence essential for viral replication of the attenuated, nonprimate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate herpesvirus, from which at least a portion of a repeat sequence has been deleted. The sequence essential for viral replication of the attenuated, nonprimate herpesvirus may be derived from a naturally-occurring nonprimate herpesvirus.

55 The deleted portion of the repeat sequence may include a portion of a repeat sequence other than a junction region or may include a junction region. Additionally, the deleted portion of the repeat sequence may comprise a nonessential sequence of one repeat sequence or both repeat sequences. Furthermore at least a portion of the essential sequence of a repeat may be deleted. In one embodiment of the invention, one entire repeat may be deleted. Moreover, a sequence not located within a repeat may additionally be deleted. In one embodiment of the invention the deleted sequence not located within a repeat is at least a portion of a gene.

The attenuated nonprimate herpesvirus may comprise DNA at least a portion of which is present in a sequence

5 essential for replication of a naturally-occurring alpha-herpesvirus. The alpha-herpesvirus may be a pseudorabies virus, infectious bovine rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I. Additionally, the alpha-herpesvirus may be a class D herpesvirus. The class D herpesvirus may be a pseudorabies virus, infectious bovine rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I. In one embodiment of the invention, the alpha-herpesvirus is an infectious bovine rhinotracheitis virus. In another embodiment of the invention, the attenuated, nonprimate herpesvirus comprises an infectious bovine rhinotracheitis virus from which has been deleted at least a portion of both repeat sequences. This virus has been constructed, designated S-IBR-002, and deposited with the ATCC under Accession No. VR 2140.

10 Further provided is an attenuated, hybrid, nonprimate herpesvirus comprising DNA which includes a sequence essential for viral replication of the attenuated, hybrid, nonprimate herpesvirus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring nonprimate, herpesvirus and at least one foreign DNA sequence. The sequence essential for viral replication of the attenuated, hybrid, nonprimate virus may be derived from a naturally-occurring nonprimate herpesvirus. Furthermore, at least a portion of a repeat sequence of the attenuated, hybrid, nonprimate herpesvirus may be deleted.

15 The foreign DNA sequence may be adapted for expression in a host and encode an amino acid sequence. Additionally, the foreign DNA sequence may be adapted for expression by a herpesvirus promoter. The herpesvirus promoter may be an endogenous upstream promoter or an inserted upstream herpesvirus promoter. The herpesvirus promoter may be the herpes simplex type ICP4 protein promoter, the herpes simplex type I thymidine kinase promoter, the pseudorabies immediate early gene promoter, the pseudorabies glycoprotein X promoter or the pseudorabies glycoprotein 92 promoter.

20 The amino acid sequence encoded by the foreign DNA sequence may be a polypeptide. Additionally the polypeptide may be a protein. Furthermore the protein, when expressed in a host, may be antigenic. In one embodiment of the invention the protein is swine rotavirus glycoprotein 38. In another embodiment, the protein is bovine rotavirus glycoprotein 38. In a further embodiment of the invention, the protein is swine parvovirus B capsid protein.

25 The attenuated, hybrid, nonprimate herpesvirus may comprise DNA, at least a portion of which is present in a sequence essential for replication of a naturally-occurring alpha-herpesvirus. The alpha-herpesvirus may be a pseudorabies virus, infectious bovine rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I. Additionally, the alpha-herpesvirus may be a class D herpesvirus. The class D herpesvirus may be pseudorabies virus, infectious bovine rhinotracheitis virus, equine herpesvirus I, feline herpesvirus I or canine herpesvirus I.

30 Furthermore the attenuated, hybrid, nonprimate herpesvirus may comprise DNA, at least a portion of which is present in a sequence essential for replication of a naturally-occurring gamma-herpesvirus. The gamma-herpesvirus may be Marek's disease virus or herpesvirus of turkeys. Additionally the gamma-herpesvirus may be a class E herpesvirus. The class E herpesvirus may be Marek's disease virus or herpesvirus of turkeys.

35 The present invention also provides a vaccine useful for immunizing an animal against a herpesvirus disease. This vaccine comprises an effective immunizing amount of a hybrid, nonprimate herpesvirus of the present invention and a suitable carrier, e.g., a physiologically balanced culture medium containing stabilizing agents.

Also provided is a multivalent vaccine useful for immunizing an animal against at least one pathogen. This vaccine comprises an effective immunizing amount of a hybrid, nonprimate herpesvirus of the present invention which includes a foreign DNA sequence encoding a protein which, when expressed in the host, is antigenic and a suitable carrier.

40 Furthermore, the present invention provides a vaccine useful for immunizing an animal against a herpesvirus disease which comprises an effective immunizing amount of an attenuated, nonprimate herpesvirus provided by the invention and a suitable carrier. Another vaccine useful for immunizing an animal against a herpesvirus disease is also provided. This vaccine comprises an effective immunizing amount of an attenuated, hybrid, nonprimate herpesvirus of the present invention and a suitable carrier.

45 Moreover, a multivalent vaccine useful for immunizing an animal against at least one pathogen is provided. This vaccine comprises an effective immunizing amount of an attenuated, hybrid, nonprimate herpesvirus which includes at least one foreign DNA sequence encoding a protein which, when expressed in the host, is antigenic and a suitable carrier.

50 Methods of immunizing animals against herpesvirus diseases and methods of immunizing an animal against at least one pathogen are provided. These methods comprise administering to the animal a suitable dose of a vaccine of the present invention. The animals which may be immunized include, but are not limited to, swine, bovine animals, sheep, goats, dogs and cats.

The vaccine may be administered by intramuscular, subcutaneous, intraperitoneal or intravenous injection. The vaccine may also be administered intranasally or orally.

55 Methods of identifying the hybrid, nonprimate herpesviruses are provided. In one embodiment of the invention, the foreign DNA sequence in the virus is detected. In another embodiment of the invention, the presence of the expressed polypeptide in the host animal or host cell is detected. In yet another embodiment of the invention, the presence of the expressed protein in the host animal or host cell is detected.

Furthermore, methods of identifying an attenuated, hybrid, nonprimate herpesvirus of the invention are provided.

In one embodiment of the invention, the foreign DNA sequence is detected. In another embodiment of the invention, the presence of the expressed polypeptide in the host animal or host cell is detected. In yet a third embodiment of the invention, the presence of the expressed protein in the host animal or host cell is detected.

The present invention further provides a method of producing in an animal a gene product for purposes other than immunization. This method comprises administering to the animal a suitable quantity of a hybrid, nonprimate herpesvirus of the present invention which includes a foreign DNA sequence adapted for expression in a host, the foreign DNA sequence of which expresses the gene product. Additionally, a gene product may be produced in an animal for purposes other than immunization by administering to the animal a suitable quantity of an attenuated, hybrid, nonprimate herpesvirus which includes a foreign DNA sequence adapted for expression in a host, the foreign DNA sequence of which expresses the gene product.

Methods of preparing an attenuated, hybrid, nonprimate herpesvirus of the present invention are also provided. One method comprises isolating naturally-occurring nonprimate herpesvirus viral DNA and using restriction enzyme digestion to produce DNA restriction fragments. These restriction fragments are purified by agarose gel electrophoresis to obtain specific DNA fragments which are treated with appropriate enzymes, known to those skilled in the art, to produce modified viral DNA fragments. These modified DNA fragments are capable of binding to bacterial plasmid DNA sequences. Suitable bacterial plasmids are separately treated with appropriate restriction enzymes, known to those skilled in the art, to produce bacterial plasmid DNA sequences capable of binding to modified viral DNA fragments. These bacterial plasmid sequences are then combined with the modified viral DNA fragments under suitable conditions to allow the viral DNA to bind the bacterial DNA and form a viral-bacterial plasmid.

The viral-bacterial DNA plasmid is then mapped by restriction enzymes to generate a restriction map of the viral DNA insert. The viral-bacterial DNA plasmid is then treated with a restriction enzyme known in the art to cause at least one deletion in the viral DNA sequence of the viral-bacterial DNA plasmid. This plasmid, containing at least one deletion in the viral DNA sequence, is transfected with naturally-occurring nonprimate herpes viral DNA into animal cells. The animal cells are maintained under suitable conditions to allow the naturally-occurring nonprimate herpesviral DNA to regenerate herpesviruses and a small percent of viruses which have recombined with the viral-foreign DNA sequence of the viral-bacterial-foreign DNA plasmid. Some of these recombined viruses have deletions in their genome as a result of deletions in the viral DNA insert of the plasmid. The viruses are identified and subsequently plaque purified away from the undesired viruses.

In another embodiment of the invention, naturally-occurring nonprimate herpes viral DNA is isolated and digested with appropriate restriction enzymes to produce viral restriction fragments. Separately, foreign DNA is digested with appropriate enzymes to produce foreign DNA restriction fragments. The foreign DNA restriction fragments are mixed with the viral DNA restriction fragments under suitable conditions so as to allow the fragments to join together to produce viral-foreign DNA fragments. Animal cells are transfected with the viral-foreign DNA fragments and maintained under suitable conditions so as to allow the foreign DNA fragments to regenerate herpesviruses and a small percent of viruses which have included foreign DNA fragments into their genome. Herpesviruses which have included desired foreign DNA fragments into their genome are identified and plaque purified away from undesired herpesviruses.

The present invention also concerns an attenuated pseudorabies virus comprising DNA which includes a sequence essential for replication of the attenuated virus, at least a portion of which is essential for replication of a naturally-occurring pseudorabies virus, and at least one foreign DNA sequence adapted for expression in a host and encoding an amino acid sequence which is antigenic in the host. In one embodiment of the invention the amino acid sequence encoded by the foreign DNA sequence is expressed in the host, such expression resulting in the production of antibodies directed to the amino acid sequence, and such antibodies conferring protection on the host against subsequent infection by a pathogen other than pseudorabies virus.

The DNA sequence which is essential for replication of the attenuated virus may be synthetic or it may be derived from a naturally-occurring pseudorabies virus. The amino acid sequence encoded by the foreign DNA sequence may be a polypeptide. In one embodiment of the invention, the polypeptide is a protein. Furthermore, the foreign DNA sequence may be adapted for expression by an endogenous upstream pseudorabies virus promoter or by an inserted upstream herpesvirus promoter. In one embodiment of the invention, the herpesvirus promoter is a pseudorabies virus promoter.

The attenuated pseudorabies virus may comprise a pseudorabies virus from which a nonessential sequence has been deleted. The deleted, nonessential sequence may be at least a portion of a gene. In one embodiment of the invention, the deleted, nonessential sequence comprises at least a portion of the pseudorabies glycoprotein X gene. In another embodiment of the invention, the foreign DNA sequence is inserted into the DNA where the deletion in the glycoprotein X gene occurred. In a specific embodiment of the invention, the foreign DNA sequence is adapted for expression by the pseudorabies glycoprotein X gene promoter and the pseudorabies glycoprotein X polyadenylation signal sequences and encodes *E. coli* beta-galactosidase. This virus has been designated S-PRV-014 and is deposited with the ATCC under Accession No. VR 2135.

In yet another embodiment of the invention, the attenuated pseudorabies virus comprises a pseudorabies virus from which has been deleted at least a portion of the glycoprotein X gene and at least a portion of a repeat sequence,

and into which has been inserted where the deletion in the glycoprotein X gene occurred a foreign DNA sequence encoding *E. coli* beta-galactosidase adapted for expression by the glycoprotein X gene promoter and polyadenylation signal sequences. In a specific embodiment of the invention, a portion of both repeat sequences is deleted. This virus is designated S-PRV-016 and has been deposited with the ATCC under Accession No. VR 2136.

5 In another embodiment of the invention, the attenuated pseudorabies virus comprises a pseudorabies virus from which at least a portion of a gene and from which at least a portion of a repeat sequence has been deleted. In one embodiment of the invention, the gene encodes pseudorabies thymidine kinase. Furthermore, the deleted sequence which comprises at least a portion of a repeat sequence may include a portion of a unique sequence. In a further embodiment of the invention, the deleted sequence which comprises at least a portion of a repeat sequence includes 10 a portion of the junction region of the internal repeat and the unique long sequence.

Furthermore, the foreign DNA sequence may be inserted into the DNA where the deletion in the repeat sequence has occurred. In one embodiment of the invention, the virus has a deletion in the pseudorabies thymidine kinase gene, a deletion of at least a portion of a repeat sequence comprising a portion of the junction region of the internal repeat 15 and a portion of the unique long sequence, and a foreign DNA sequence inserted where the deletion in the junction region occurred. In a specific embodiment of the invention, the foreign DNA sequence is adapted for expression by the herpes simplex type I ICP4 gene promoter and encodes herpes simplex virus type I thymidine kinase. This virus is designated S-PRV-004.

The invention also concerns attenuated pseudorabies viruses from which have been deleted at least a portion of 20 the thymidine kinase gene and at least a portion of both repeat sequences. In one embodiment of the invention, a foreign DNA sequence is inserted into the virus where the deletions in both repeat sequences occurred. In another embodiment of the invention, the virus has a deletion in the pseudorabies thymidine kinase gene, a deletion in both 25 repeat sequences, and an insertion of a foreign DNA sequence into both repeats. In a specific embodiment of the invention, the foreign DNA sequence is adapted for expression by the herpes simplex type I ICP4 gene promoter and encodes herpes simplex virus type I thymidine kinase. This virus is designated S-PRV-005 and has been deposited with the ATCC under Accession No. VR 2109. In another specific embodiment of the invention, the foreign DNA sequence 30 is adapted for expression by the herpes simplex type I ICP4 gene promoter and encodes swine rotavirus glycoprotein 38. This virus is designated S-PRV-007 and is deposited under ATCC Accession No. VR 2118. In yet a further specific embodiment of the invention, the foreign DNA is adapted for expression by the herpes simplex type I thymidine kinase gene promoter and encodes *E. coli* beta-galactosidase (the lacZ gene). This virus is designated S-PRV-010 and has been deposited under ATCC Accession No. VR 2110.

In another embodiment of the invention, at least a portion of each repeat sequence, the gene encoding pseudorabies thymidine kinase and the gene encoding pseudorabies glycoprotein X have been deleted. Furthermore, the foreign DNA sequence may be inserted into the DNA sequence where the deletion in the pseudorabies glycoprotein X gene occurred. In a specific embodiment of the invention, the foreign DNA sequence encodes an antigenic portion of the 35 swine parvovirus B capsid protein and is adapted for expression by the pseudorabies glycoprotein X gene promoter and the pseudorabies glycoprotein X polyadenylation signal sequences. This virus, designated S-PRV-020, has been deposited under ATCC Accession No. VR 2137.

In yet a further embodiment of the invention, the virus has a deletion in the pseudorabies thymidine kinase gene, a deletion in both repeat sequences and insertions of foreign DNA sequences which encode an antigenic portion of the 40 swine parvovirus B capsid protein into both repeat sequences. The foreign DNA sequences are adapted for expression by the pseudorabies glycoprotein X gene promoter and the pseudorabies glycoprotein X polyadenylation signal sequences. This virus, designated S-PRV-025, has been deposited under ATCC Accession No. VR 2138.

In still another embodiment of the invention, at least a portion of a repeat sequence has been deleted from a pseudorabies virus and at least a portion of the pseudorabies glycoprotein X gene has been deleted. The deleted sequence 45 which comprises at least a portion of a repeat sequence may include a portion of a unique sequence. In one embodiment of the invention, the deleted sequence which comprises at least a portion of a repeat sequence and a portion of a unique sequence comprises a portion of the junction region of the internal repeat and the unique long sequence. Furthermore, the foreign DNA sequence may be inserted into the DNA sequence where the deletion in glycoprotein X gene occurred. In a specific embodiment of the invention, the foreign DNA sequence is adapted for expression by the pseudorabies glycoprotein X promoter and the pseudorabies glycoprotein X polyadenylation signal sequences and encodes 50 *E. coli* beta-galactosidase. This virus, designated S-PRV-029, has been deposited under ATCC Accession No. VR 2139.

Vaccines useful for immunizing an animal against pseudorabies virus disease are also provided. These vaccines 55 comprise an effective immunizing amount of an attenuated pseudorabies virus of the present invention and a suitable carrier, e.g. a physiologically balanced culture medium which contains stabilizing agents.

The invention further concerns multivalent vaccines useful for immunizing an animal against pseudorabies virus disease and at least one other pathogen. The host animal is immunized against other pathogens by the production within the animal of foreign antigens encoded by the foreign DNA sequence of the attenuated pseudorabies virus. Animals may be immunized against pseudorabies virus and other pathogens by administering to them a suitable dose of

a vaccine of the present invention. Such animals include swine, dogs, cats, sheep or bovine animals. The vaccine may be administered by intramuscular, subcutaneous, intraperitoneal or intravenous injection. The vaccine may also be administered intranasally or orally.

The invention also provides methods of preparing attenuated pseudorabies viruses which include a foreign DNA sequence. One method involves isolating naturally-occurring pseudorabies viral DNA and using restriction enzyme digestion to produce DNA restriction fragments. These restriction fragments are purified by agarose gel electrophoresis to obtain specific DNA fragments which are treated with appropriate enzymes, known to those skilled in the art, to produce modified viral DNA fragments. These modified viral DNA fragments are capable of binding to bacterial plasmid DNA sequences. Suitable bacterial plasmids are separately treated with appropriate restriction enzymes, known to those skilled in the art, to produce bacterial plasmid DNA sequences capable of binding to modified viral DNA fragments. These bacterial plasmid DNA sequences are then combined with the modified viral DNA fragments under suitable conditions to allow the viral DNA to bind to the bacterial DNA and form a viral-bacterial plasmid.

The viral-bacterial DNA plasmid is then mapped by restriction enzymes to generate a restriction map of the viral DNA insert. The viral-bacterial DNA plasmid is then treated with restriction enzymes known in the art to cut the viral DNA sequence of the viral-bacterial DNA plasmid. Foreign DNA is separately digested with appropriate restrictive enzymes to produce foreign DNA restriction fragments. Fragments including desired genes are mixed with the viral-bacterial DNA plasmid sequences under suitable conditions to allow the formation of a viral-bacterial-foreign DNA plasmid. This plasmid, containing the foreign DNA sequence, is transfected with naturally-occurring intact pseudorabies viruses into animal cells. The animal cells are maintained under suitable conditions to allow the naturally-occurring pseudorabies viral DNA to regenerate the naturally-occurring virus and a small percentage of viruses which have recombined with the foreign DNA sequence of the plasmid. Some of these recombined viruses have deletions in their genome as a result of deletions in the viral DNA insert of the plasmid. Viruses including the foreign DNA sequence are identified and subsequently plaque purified away from the wild type virus.

The foreign DNA sequence incorporated into the pseudorabies viral genome may encode herpes simplex type I thymidine kinase, *E. coli* beta-galactosidase, swine rotavirus glycoprotein 38 or swine parvovirus B capsid protein.

Another method of preparing attenuated pseudorabies viruses which include a foreign DNA sequence is provided. This direct ligation method involves isolating and digesting pseudorabies viral DNA with appropriate restriction enzymes to produce viral DNA restriction fragments. Foreign DNA is separately digested with restriction enzymes to produce foreign DNA restriction fragments. The viral DNA restriction fragments are reacted under suitable conditions to allow the formation of viral-foreign DNA fragments.

Animal cells are then transfected with the viral-foreign DNA fragments and maintained under suitable conditions to allow the viral-foreign DNA fragments to regenerate pseudorabies viruses. Viruses which include desired foreign DNA sequences are identified and plaque purified away from the other viruses.

In one embodiment of the invention, the direct ligation method involves incorporating into the pseudorabies viral genome a foreign DNA sequence encoding *E. coli* beta-galactosidase.

The invention further provides methods of preparing vaccines comprised of pseudorabies viruses which include a foreign DNA sequence. These methods include cultivating the virus in roller bottles or in a suspension of microcarrier beads. The vaccines can also be prepared by cultivating the virus by batch fermentation.

The invention also provides an isolated nucleic acid molecule having the nucleic acid sequence set forth in Figure 10 and encoding swine rotavirus glycoprotein 38. This nucleic acid molecule may be a cDNA molecule. The invention further concerns a mRNA molecule which is complementary to the nucleic acid molecule set forth in Figure 10.

A bacterial recombinant cloning vehicle which comprises plasmid DNA and the cDNA of this invention is also provided. In a specific embodiment of the invention, the plasmid DNA comprises pBR322 DNA. This cloning vehicle is designated pSY565. A bacterial host cell which comprises this cloning vehicle is additionally provided. In a specific embodiment, the host cell is an *E. coli* cell designated DH-1/pSY565 deposited under ATCC Accession No. 53,340.

Further provided is an isolated nucleic acid molecule having the nucleic acid sequence set forth in Figure 11 and encoding swine parvovirus B capsid protein. This nucleic acid molecule may be a cDNA molecule. The invention also provides a mRNA molecule which is complementary to the nucleic acid molecule set forth in Figure 11.

Moreover, a bacterial recombinant cloning vehicle which comprises plasmid DNA and the DNA of Figure 11 is also provided. In a specific embodiment of the invention, the plasmid DNA comprises pSP64 DNA. This cloning vehicle is designated pSY875. A bacterial host cell which comprises this cloning vehicle is additionally provided. In a specific embodiment, the host cell is an *E. coli* cell designated HB101/pSY875 deposited under ATCC Accession No. 67146.

The present invention further provides an attenuated pseudorabies virus comprising DNA which includes a sequence essential for replication of the attenuated pseudorabies virus, at least a portion of which is present in a sequence essential for replication of a naturally-occurring pseudorabies virus. This DNA encodes mRNAs which, in an animal infected with the attenuated pseudorabies virus, are translated into antigenic pseudorabies virus gene products which invoke in the infected animal an immunological response distinguishable from an immunological response invoked in an animal infected with a naturally-occurring pseudorabies virus. Within this application, a naturally-occurring pseudorabies virus means a pseudorabies virus which has not been genetically engineered, and includes, but is not

limited to, wild-type pseudorabies viruses and pseudorabies viruses selected from pseudorabies viruses which exist in nature and have spontaneous deletions.

The sequence essential for replication of the attenuated pseudorabies virus may be derived from a naturally-occurring pseudorabies virus. Additionally the DNA may comprise wild-type pseudorabies viral DNA from which at least a portion of a nonessential gene has been deleted. Within this application, nonessential gene means a gene which is not essential for viral replication.

In one embodiment of the invention, the nonessential gene of which at least a portion has been deleted is the gpX gene. In another embodiment of the invention, substantially all of the gpX gene coding region, a portion of a repeat sequence and the thymidine kinase gene are deleted. In yet another embodiment of the invention, substantially all of the gpX gene coding region, a portion of a repeat sequence and the thymidine kinase gene are deleted and the herpes simplex virus-1 (HSV-1) thymidine kinase gene under the control of the ICP4 promoter is inserted in place of the deleted gpX gene coding region. This virus, designated S-PRV-012, has been deposited with the ATCC under Accession No. VR 2119.

In still a further embodiment of the invention, the gpX gene coding region, a portion of a repeat sequence and the thymidine kinase gene are deleted and the *E. coli* beta-galactosidase gene is inserted in place of the deleted gpX gene coding region. The inserted beta-galactosidase gene is under the control of the endogenous gpX promoter. This virus, designated S-PRV-013, has been deposited with the ATCC under Accession No. VR 2120.

The present invention further provides a vaccine for pseudorabies virus disease which comprises an effective immunizing amount of an attenuating pseudorabies virus of the present invention and a suitable carrier. The suitable carrier may be a physiologically balanced culture medium containing stabilizing agents. Additionally, the effective immunizing amount, i.e. an amount necessary to invoke the production of antibodies by the animal which confer protection on the animal against subsequent infection by wild-type pseudorabies virus, may be from about  $10^3$  to about  $10^6$  plaque forming units (PFU)/dose. Moreover, the effective immunizing amount may be from about  $10^4$  to about  $10^5$  PFU/dose.

In one embodiment of the invention, the vaccine comprises an effective immunizing amount of the attenuated pseudorabies designated S-PRV-012 and a suitable carrier. In yet another embodiment, the vaccine comprises an effective immunizing amount of the attenuated pseudorabies virus designated S-PRV-013 and a suitable carrier.

The present invention also provides a method of immunizing an animal against pseudorabies virus disease. This method comprises administering to the animal a suitable dose of a vaccine of the present invention. In one embodiment of the invention, the effective immunizing amount of the attenuated pseudorabies virus of the vaccine is from about  $10^3$  to about  $10^6$  PFU/dose. Moreover, the animal may be a swine. In another embodiment of the invention, the effective immunizing amount of the attenuated pseudorabies virus of the vaccine is from about  $10^4$  to about  $10^5$  PFU/dose. The animal immunized by this method may be a swine, dog, cat, sheep or bovine animal. In yet another embodiment of the invention, an animal may be immunized against pseudorabies virus disease by administering to the animal a suitable dose of a vaccine of the present invention which comprises the attenuated pseudorabies virus S-PRV-012. The immunized animal may be a swine. Still another method of immunizing an animal against pseudorabies virus disease comprises administering to the animal a suitable dose of the vaccine of the present invention which comprises the attenuated pseudorabies virus S-PRV-013. Furthermore, the immunized animal may be a swine.

The present invention also provides a method for distinguishing an animal vaccinated with a vaccine of the present invention from an animal infected with a naturally-occurring pseudorabies virus. This method comprises analyzing a body fluid of the animal for the presence of antigens normally expressed in an animal infected with a naturally-occurring pseudorabies virus, identifying antigens which are present in the body fluid and antigens which are not present in the body fluid and correlating said antigens which are not present in the body fluid with antigens which are not expressed in an animal infected with the attenuated pseudorabies virus of the vaccine. The presence in the body fluid of antigens which are normally expressed in the animal by a naturally-occurring pseudorabies virus except for those antigens which are not expressed in the animal by the attenuated pseudorabies virus of the vaccine would be indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring pseudorabies virus.

In one embodiment of the invention the presence of antigens in the body fluid is determined by detecting in the body fluid antibodies specific for the antigens.

Also provided is a method for distinguishing an animal vaccinated with a vaccine comprising the attenuated pseudorabies virus S-PRV-012 from an animal infected with a naturally-occurring pseudorabies virus. According to this method, a body fluid of the animal is analyzed for the presence of glycoprotein X and at least one other antigen normally expressed in the animal by a naturally-occurring pseudorabies virus. Antigens which are present in the body fluid are identified and the presence or absence of glycoprotein X in the body fluid is determined. The presence of antigens which are normally expressed in the animal by a naturally-occurring pseudorabies virus and the absence of glycoprotein X in the body fluid would be indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring pseudorabies virus. In another embodiment of the invention the presence of antigens and glycoprotein X in the body fluid is determined by detecting in the body fluid antibodies specific for the antigens and glycoprotein X.

The present invention further provides a method for distinguishing an animal vaccinated with a vaccine which comprises the attenuated pseudorabies virus S-PRV-013 from an animal infected with a naturally-occurring pseudorabies

virus. This method comprises analyzing a body fluid of the animal for the presence of glycoprotein X and at least one other antigen normally expressed in an animal by a naturally-occurring pseudorabies virus. Antigens which are present in the body fluid are identified and the presence or absence of glycoprotein X in the body fluid is determined. The presence of antigens which are normally expressed in an animal by a naturally-occurring pseudorabies virus and the absence of glycoprotein X in the body fluid would be indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring pseudorabies virus. The presence of the antigens and glycoprotein X may be determined by detecting in the body fluid antibodies specific for the antigens and glycoprotein X.

Furthermore, the present invention provides a method for preparing a pseudorabies virus vaccine of the present invention. In one embodiment of the invention, the attenuated pseudorabies virus is cultured in roller bottles. In another embodiment of the invention, the attenuated pseudorabies virus is cultivated in a suspension of microcarrier beads. In yet another embodiment of the invention, the attenuated pseudorabies virus is cultivated by batch fermentation.

Methods for constructing, selecting and purifying herpesviruses of the present invention, as well as assay techniques for determining the immune status of swine following vaccination and challenge with wild-type pseudorabies viruses, are detailed in the following Materials and Methods and Examples sections, which illustrate, but in no way limited, the scope of the subject invention, which is defined by the claims.

#### MATERIALS AND METHODS

GROWTH OF HERPESVIRUS IN TISSUE CULTURE. All of the herpesviruses under discussion were grown in tissue culture cells. Unless otherwise noted, the cells used were: Vero cells for PRV; MDBK cells for IBR; CEF cells for HVT; Crandall feline kidney cells for FHV. Vero cells are suitable for EHV and MDCK cells are suitable for CHV.

PREPARATION OF HERPESVIRUS STOCK SAMPLES. Herpesvirus stock samples were prepared by infecting tissue culture cells at a multiplicity of infection of 0.01 PFU/cell in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin (these components were obtained from Irvine Scientific or equivalent supplier, and hereafter are referred to as complete DME medium) plus 1% fetal bovine serum. After cytopathic effect was complete, the medium and cells were harvested and the cells were pelleted at 3000 rpm for 5 minutes in a clinical centrifuge. For all herpesviruses except HVT, the cells were resuspended in 1/10 the original volume of medium, and an equal volume of 2 times autoclaved skim milk (9% skim milk powder in H<sub>2</sub>O wgt/vol) was added. The virus sample was frozen and thawed 2 times, aliquoted, and stored frozen at -70°C. The titer was usually about 10<sup>8</sup> plaque forming units per ml. For HVT, infected cells were resuspended in complete medium containing 20% fetal bovine serum, 10% DMSO and stored frozen at -70°C.

PREPARATION OF HERPESVIRUS DNA. For herpesvirus DNA preparation, a confluent monolayer of tissue culture cells in a 25 cm<sup>2</sup> flask or a 60 mm petri dish was infected with 100 microliters of virus sample in 1 ml medium. Adsorption proceeded for 1-2 hours at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air. After adsorption, 4 mls of complete DME medium plus 1% fetal bovine serum were added. After overnight incubation, or when the cells were showing 100% cytopathic effect, the cells were scraped into the medium with a cell scraper (Costar brand). The cells and medium were centrifuged at 3000 rpm for 5 minutes in a clinical centrifuge. The medium was decanted, and the cell pellet was gently resuspended in 0.5 ml solution containing 0.01 M Tris pH 7.5, 1 mM EDTA, and 0.5% Nonidet P-40 (NP40, an ionic detergent comprising an octyl phenol ethylene oxide condensate containing an average of 9 moles ethylene oxide per molecule, purchased from Sigma Chemical Co., St. Louis, MO.). The sample was incubated at room temperature for 10 minutes. Ten microliters of a stock solution of RNase A (Sigma) were added (stock was 10 mg/ml, boiled for 10 minutes to inactivate DNAase). The sample was centrifuged for 5 minutes at 3000 rpm in a clinical centrifuge to pellet nuclei. The DNA pellet was removed with a pasteur pipette or wooden stick and discarded. The supernatant fluid was decanted into a 1.5 ml Eppendorf tube containing 25 microliters of 20% sodium dodecyl sulfate (Sigma) and 25 microliters proteinase-K (10 mg/ml; Boehringer Mannheim supplier). The sample was mixed and incubated at 37°C for 30-60 minutes. An equal volume of water-saturated phenol was added and the sample was mixed on a vortex mixer for 1 minute. The sample was centrifuged in an Eppendorf minifuge for 5 minutes at full speed. The upper aqueous phase was removed to a new Eppendorf tube, and two volumes of -20°C absolute ethanol were added and the tube put at -20°C for 30 minutes to precipitate nucleic acid. The sample was centrifuged in an Eppendorf centrifuge at 4°C for 5 minutes. The supernatant was decanted, and the pellet was washed one time with cold 80% ethanol. The pellet was dried in a lyophilizer, and rehydrated in 17 microliters H<sub>2</sub>O. For the preparation of larger amounts of DNA, the procedure was scaled up to start with a 850 cm<sup>2</sup> roller bottle of Vero cells. The DNA was stored in H<sub>2</sub>O or in 0.01 M Tris pH 7.5, 1 mM EDTA at -20°C or +4°C.

PHENOL EXTRACTION. Phenol extraction was performed on any convenient volume of DNA sample, typically between 100 microliters to 1 ml. The DNA sample was diluted in 0.01M Tris pH 7.5, 1 mM EDTA and an equal volume of water saturated phenol was added. The sample was mixed briefly on a vortex mixer and placed on ice for 3 minutes. After centrifugation for 3 minutes in a microfuge, the aqueous layer was removed to a new tube and was precipitated by ethanol.

ETHANOL PRECIPITATION. DNA in a sample was concentrated by ethanol precipitation. To the DNA sample were

added 1/10 volume of 3M sodium acetate, pH 7.5 and 3 volumes of cold ethanol. The DNA was precipitated for 30 minutes at -70°C or overnight at -20°C and then pelleted by centrifugation in the microfuge for 15 minutes at 4°C. The pellet was washed once with 200 microliters of cold 80% ethanol and pelleted again for 10 minutes at 4°C. After air drying or lyophilization, the pellets were resuspended in the appropriate buffer or H<sub>2</sub>O.

5 RESTRICTION ENZYME DIGESTION. DNA was cut by restriction enzymes using the buffer recommended by the manufacturer (International Biotechnologies Inc., New Haven, CT. (IBI), Bethesda Research Laboratories, Bethesda, MD. (BRL), and New England Biolabs, Beverly, MA). Whenever possible, the concentration of DNA was kept below 1 microgram/50 microliters. Incubation was at 37°C for 1-4 hours.

10 AGAROSE GEL ELECTROPHORESIS OF DNA. To visualize the restriction pattern of the DNA, 5 microliters of loading buffer (5X electrophoresis buffer, 0.01% bromphenol blue dye, 50 mM EDTA, and 50% glycerol) were added. The sample was loaded into a lane in a horizontal submarine electrophoresis unit containing a 0.6% agarose gel. The 15 electrophoresis buffer was 40 mM Tris, 10 mM EDTA, adjusted to pH 7.8 with acetic acid, and with or without 0.5 micrograms/ml ethidium bromide. The gel was run at 40-50V for 18 hours, and the gel was removed and stained with 0.5 micrograms/ml ethidium bromide for 30 minutes. The DNA bands were visualized on a long wavelength UV transilluminator.

15 PHOSPHATASE TREATMENT OF DNA. Phosphatase treatment of DNA was performed by adding 1 microliter (25 units) of calf intestinal phosphatase (Boehringer Mannheim) directly to the restriction enzyme digestion reactions and continuing the incubation for 30 minutes at 37°C. The phosphatase was inactivated for 60 minutes at 65°C prior to phenol extraction.

20 POLYMERASE FILL-IN REACTION. DNA was resuspended in buffer containing 50 mM Tris pH 7.4, 50 mM KCl, 5 mM MgCl<sub>2</sub>, and 400 micromolar each of the four deoxynucleotides. Ten units of Klenow DNA polymerase (BRL) were added and the reaction was allowed to proceed for 15 minutes at room temperature. The DNA was then phenol extracted and ethanol precipitated as above.

25 EXONUCLEASE RESECTION REACTION. DNA was resuspended in 100 microliters of 60 mM Tris pH 8.0, 0.66 mM MgCl<sub>2</sub>, 1 mM beta-mercaptoethanol. The sample was warmed to 30°C for 5 minutes, and 10 units of lambda exonuclease III (BRL) were added. At frequent time intervals (e.g. every 2.5 minutes), 10 microliter aliquots were diluted into 100 microliters of 30 mM sodium acetate pH 4.5, 250 mM NaCl, 1 mM ZnSO<sub>4</sub>, 4 micrograms/100 microliters yeast 30 tRNA, 30 units/ 100 microliters S1 nuclease. After 45 minutes at 30°C, 15 microliters of stop buffer consisting of 625 mM Tris pH 9.0, 150 mM EDTA, 1% SDS were added. The samples were then phenol extracted and ethanol precipitated as above. The DNA digestion products were then analyzed and purified by agarose gel electrophoresis.

35 PHENOL EXTRACTION OF DNA FROM AGAROSE. DNA bands cut from low melting point agarose gels were diluted to less than 0.5% agarose to a final concentration of 0.3 M sodium acetate. The samples were heated to 65°C to melt the agarose and then cooled to 37°C for 5 minutes. An equal volume of phenol was added and the sample was phenol extracted three times (see PHENOL EXTRACTION). The DNA was then ethanol precipitated and the pellet resuspended at a concentration of 3-6 fmole DNA/ microliter.

40 LIGATION. DNA was joined together by the action of the enzyme T4 DNA ligase (BRL). Ligation reactions contained 10 fmols DNA, 20 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 200 micromolar ATP, and 20 units T4 DNA ligase in 10 microliters final reaction volume. The ligation was allowed to proceed for 3-16 hours at 15°C. Typically DNA fragments to be ligated together were added at an equal molar ratio. Typically two different DNA fragments 45 were joined during ligation, but joining of three or four different DNAs at once was also possible.

45 RESTRICTION MAPPING OF DNA. Restriction mapping of DNA was performed as detailed in Maniatis et al. (1). Once it was cloned, the DNA was digested with a number of different restriction enzymes and the DNAs were analyzed on agarose gels and the sizes of the resulting fragments were measured. A double digest with two different restriction enzymes was performed on the same DNA sample to aid in the interpretation of the maps. Another approach used was 50 to cut the DNA with a restriction enzyme that has a single unique site in the DNA, label the end of the DNA with <sup>32</sup>P using T4 DNA kinase or Klenow DNA polymerase (see POLYMERASE FILL-IN REACTION) and then cut the DNA with other restriction enzymes at low temperature or for short times so that only partial digestion occurred. The subsequent analysis of the partial digestion fragments on agarose gels served to order the restriction sites on the map. All of these mapping procedures are well understood by those skilled in the art and are detailed in Maniatis et al. (1). The most complete restriction maps can only be composed once the DNA has been sequenced, and the sequence is then analyzed by a computer searching for all the known restriction enzyme sites. Some of our maps have been generated from sequence information.

55 SOUTHERN BLOTTING OF DNA. The general procedure for Southern blotting was taken from Maniatis et al. (1). DNA was blotted to nitrocellulose filters (S&S BA85) in 20X SSC (1X SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.0), and prehybridized in hybridization solution consisting of 30% formamide, 1X Denhardt's solution (0.02% polyvinylpyrrolidone (PVP), 0.02% bovine serum albumin (BSA), 0.02% Ficoll), 6X SSC, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 6.8, 200 micrograms/ml salmon sperm DNA for 4-24 hours at 55°C. Labeled probe DNA was added that had been labelled by nick translation using a kit from Bethesda Research Laboratories (BRL) and one <sup>32</sup>P-labeled nucleotide. The probe DNA was separated from the unincorporated nucleotides by NACS column (BRL) or on a Sephadex G50 column (Phar-

macia). After overnight hybridization at 55°C, the filter was washed once with 2X SSC at room temperature followed by two washes with 0.1X SSC, 0.1% sodium dodecyl sulfate (SDS) for 30 minutes at 55°C. The filter was dried and autoradiographed.

5 DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS. The method is based upon the calcium phosphate DNA precipitation procedure of Graham and Van der Eb (32) with the following modifications. For transfection into animal cells, 0.1-0.2 micrograms of plasmid DNA containing the foreign DNA flanked by appropriate herpesvirus cloned sequences (the homovector) were mixed with 0.3 micrograms of intact DNA. Both DNAs were stored either in H<sub>2</sub>O or 0.01 M Tris pH 7.5, 1 mM EDTA and the final volume should be less than 0.25 ml. To the mixture was added an equal volume of 2X HEPES buffered saline (10g N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid (HEPES),  
10 16g NaCl, 0.74g KCl, 0.25g Na<sub>2</sub>HP0<sub>4</sub>.2H<sub>2</sub>O, 2g dextrose per liter H<sub>2</sub>O and buffered with NaOH to pH 7.4). The mixture was then diluted to 0.5 ml by the addition of the appropriate volume of 1X HEPES buffered saline (prepared by diluting the above solution 1:1 with H<sub>2</sub>O). After mixing, 35 microliters of 2.2 M CaCl<sub>2</sub> were added to the DNA mixture and mixed.

15 The mixture was incubated at room temperature for 30 minutes. Medium was removed from an 80% confluent monolayer of rabbit skin cells, Vero cells, or CEF cells growing in a 25 cm<sup>2</sup> flask, and the DNA mixture was added to the flask and distributed over the cells. After a 30 minute incubation at room temperature, 5 mls of complete DME medium plus 10% fetal bovine serum were added. The cells were incubated for 5 hours at 37°C in a humidified incubator containing 5% CO<sub>2</sub> in air. The medium was changed at 5 hours either with or without a glycerol shock. When used, the glycerol shock consisted of removing the medium and adding DME containing 20% glycerol for 3 minutes at room temperature, followed by a wash with 10% glycerol in DME, and a wash in 5% glycerol in DME, followed by the addition  
20 of fresh complete DME medium plus 10% fetal bovine serum. The cells were incubated at 37°C as above for 3-4 days until cytopathic effect from the virus was 50-100%. Virus was harvested as described above for the preparation of virus stocks. This stock was referred to as a transfection stock and it was subsequently screened for recombinant virus either with or without a selection mechanism to enrich for recombinant plaques as described below.

25 DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUSES. Rather than using homovectors and relying upon homologous recombination to generate recombinant virus, the technique of direct ligation was developed to insert foreign genes into herpesviruses. In this instance, the cloned foreign gene did not require flanking herpesvirus DNA sequences but only required that it have restriction sites available to cut out the foreign gene fragment from the plasmid vector. A compatible restriction enzyme was used to cut the herpesvirus DNA. A requirement of the technique was that the restriction enzyme used to cut the herpesvirus DNA must cut at a limited number of sites, 30 preferably less than 3 sites. For PRV DNA, we have used xbaI, which cut PRV DNA in two places, and contemplate the use of HindIII (2 cuts), EcoRV (2 or 3 cuts) or NdeI (3-5 cuts). The herpesvirus DNA was mixed with a 30-fold molar excess of plasmid DNA, and the mixture was cut with the appropriate restriction enzyme. The DNA mixture was phenol extracted and ethanol precipitated to remove restriction enzymes, and ligated together according to the ligation procedure detailed above. The ligated DNA mixture was then phenol extracted, ethanol precipitated, and resuspended in 298  
35 microliters 0.01M Tris pH 7.5, 1 mM EDTA. Forty-two microliters of 2M CaCl<sub>2</sub> were added, followed by an equal volume of 1X HEPES buffered saline (see above), and the sample was used to transfect animal cells as described above.

40 The virus in the transfection stock was then screened for foreign DNA inserts as described below. The advantage of the direct ligation technique was that it required less construction of sub-clones in the plasmid state, and that the recombinant virus was present in the transfection stock at a much higher frequency than with homologous recombination.

HAT SELECTION OF RECOMBINANT HERPESVIRUS EXPRESSING THYMIDINE KINASE. Deletion mutants of herpesviruses which suffered deletions in the thymidine kinase (TK) gene were constructed. These PRV strains have been designated S-PRV-002 and S-PRV-003 and have been deposited with the ATCC under Accession No. VR 2107 and VR 2108 respectively. These TK minus (TK-) viruses have been used as recipients for the insertion of the foreign  
45 herpes simplex type 1 (HSV-1) TK gene. One HSV-1 TK gene that we have used contains the HSV-1 ICP4 promoter and was from B. Roizman (16). It was sub-cloned to lie between two flanking regions of PRV DNA, for example by insertion of the TK gene into PRV BamHI #5 fragment between XbaI and HpaI sites. The plasmid construct was then transfected with the PRV TK- DNA to yield recombinant virus. The transfection stock was enriched for TK-containing virus by the HAT selection procedure described in (35). The transfection stock was used to infect monolayers of 143 TK- cells in  
50 60 mm culture dishes that had been preincubated in HAT medium for 16 hours at 37°C (HAT medium: medium 199 containing 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 10% fetal bovine serum, 5 X 10<sup>-5</sup> M hypoxanthine, 10<sup>-5</sup> M thymidine, 5 X 10<sup>-6</sup> M aminopterin). Samples of the transfection stock virus were infected into the 143 TK- cells using 10<sup>-3</sup> to 10<sup>-7</sup> dilutions of virus. After one or two days at 37°C, the dishes inoculated with the highest dilution of virus and still showing virus plaques were harvested for virus stocks, and the selection was repeated a second time. The virus stock harvested from the second HAT selection was used in a plaque assay and individual plaques were  
55 picked and tested for foreign DNA inserts as described below.

BROMODEOXYURIDINE SELECTION OF RECOMBINANT HERPESVIRUS. In order to insert a foreign gene in place of a TK gene already present in the herpesvirus genome, the foreign gene was cloned in plasmids so that it contained the same flanking homology regions as the TK genes. These flanking regions could be part of the TK gene itself,

or parts of the herpesvirus that flank the TK gene. In either case, the plasmid DNA containing the foreign gene was transfected with intact herpesvirus genomic DNA containing the HSV-1 TK gene. The transfection stock of recombinant virus was grown for two selections in 143 TK- cells in the presence of 40 micrograms/ml bromodeoxyuridine (BUDR, Sigma) in complete DME medium plus 10% fetal bovine serum. The drug BUDR is an analogue of thymidine that is recognized by the viral enzyme thymidine kinase (TK) and is ultimately incorporated into DNA. When incorporated into the DNA, BUDR is mutagenic and lethal and thus selects against viruses that have an active TK gene. By this selection method, viruses that had exchanged their TK gene for a foreign gene by homologous recombination were enriched in the population. Screening for the recombinant viruses was then performed by one of the techniques detailed below.

HYBRIDIZATION SCREEN FOR RECOMBINANT HERPESVIRUS. One procedure used is described in (36). The technique involved doing a plaque assay on PRV under agarose, removing the agarose once plaques had formed, and lifting the cell monolayer from the dish onto a nitrocellulose membrane filter. The filter was then processed through the Southern procedure for DNA hybridization as detailed above. The DNA probe used in the procedure was made from the foreign gene that had been inserted into the virus. Thus plaques that contain the foreign gene were identified, and they were picked from the agarose overlay that had been saved.

BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS. When the foreign gene encoded the enzyme beta-galactosidase, the plaques that contained the gene were visualized more easily. The chemical Bluogal™ (BRL) was incorporated at the level of 200-300 micrograms/ml into the agarose overlay during the plaque assay, and the plaques that expressed active beta-galactosidase turned blue. The blue plaques were then picked and purified by further blue plaque isolations. Other foreign genes were inserted by homologous recombination such that they replaced the beta-galactosidase gene; in this instance non-blue plaques were picked for purification of the recombinant virus.

ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS. A third method for screening the recombinant virus stock was to look directly for the expression of the foreign gene with antibodies. Herpesvirus plaques were spotted and picked by inserting a toothpick through the agarose above the plaque and scraping the plaque area on the dish. Viruses were then rinsed from the toothpick by inserting the toothpick into a well of a 96-well microtiter dish (Falcon Plastics) containing a confluent monolayer of tissue culture cells that had been washed 3 times in DME medium without serum. It was important for the virus to grow without serum at this stage to allow the immunological procedure to work. After cytopathic effect was complete, the plates were put at -70°C to freeze and lyse the cells. The medium was thawed, and the freeze/thaw procedure was repeated a second time. Then 50-100 microliters of medium were removed from each well and filtered under vacuum through a nitrocellulose membrane (S&S BA85) using a DotBlot™ apparatus (BRL). The filter blots were soaked in a blocking solution of 0.01 M Tris pH 7.5, 0.1 M NaCl, 3% bovine serum albumin at room temperature for two hours with shaking. The filter blots were then placed in a sealable bag (Sears Seal-A-Meal® or equivalent), and 10 mls of the blocking solution that contained 10 microliters of antibody specific for the foreign protein were added. After overnight incubation at room temperature with shaking, the blot was washed 3 times with 100 mls 0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.05% Tween 20 detergent (Sigma). The blot was put in another sealable bag and 10 mls blocking solution containing  $10^6$  counts per minute of  $^{125}\text{I}$ -protein A (New England Nuclear) were added. After allowing the protein A to bind to the antibody for 2 hours at room temperature with shaking, the blot was washed as above, dried, and overlayed with an x-ray film and an intensifying screen (Dupont) and autoradiographed for 1-3 days at -70°C. The film was developed by standard procedures. Virus from the positive wells which contained the recombinant virus was further purified.

WESTERN BLOTTING PROCEDURE. Samples of cell lysates, positive controls and protein standards were run on a polyacrylamide gel according to the procedure of Laemmli (42). After electrophoresis, the gel was soaked in a transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol) plus 0.1% SDS for 20 minutes. The stacking gel portion was removed and the separation gel was placed onto Whatman 3 mm paper. A matching-sized piece of nitrocellulose filter was prewet in the transfer buffer and placed onto the polyacrylamide gel to cover the gel completely and make intimate contact. A prewet piece of Whatman 3 mm paper was placed on top of the nitrocellulose filter to create a "sandwich", and the sandwich was placed into an electrophoretic transfer device (Biorad). The sandwich was completely submerged in transfer buffer. The electrophoretic transfer was carried out for 3 hours at 250 millamps. After transfer, the nitrocellulose filter was removed from the assembly and placed in a dish containing 50 mls of blocking buffer (50 mg/ml bovine serum albumin, 10 mM magnesium chloride, 100 mM potassium chloride, 1 mM calcium chloride, 10 mM imidazole pH 7.0, 0.3% Tween-20, 0.02% sodium azide). The nitrocellulose blot was incubated for 1-2 hours in the blocking buffer at room temperature on a shaker. The blot was then placed in a sealable bag containing 15 mls of the blocking buffer plus the specific antiserum as a probe and incubated overnight at 37°C on a shaker. The blot was then removed from the probe solution and rinsed with 5-6 changes of phosphate buffered saline over a period of 1 hour. The phosphate buffered saline was removed and 50 mls of blocking buffer containing  $5 \times 10^5$  cpm of  $^{125}\text{I}$  labeled protein A (Amersham) were added. The blot was incubated for 1 hour with the labeled protein A solution, the labeled protein A solution was removed and the blot was rinsed with 5-6 changes of phosphate buffered saline solution containing 0.3% Tween-20. The blot was air dried and autoradiographed overnight with an intensifying screen.

METHOD FOR cDNA CLONING SWINE ROTAVIRUS gp38 GENE Virus Growth. The OSU strain of porcine rotavirus (ATCC VR-892) was propagated on MA-104 cells (Rhesus monkey kidney cells from MA Bioproducts). Confluent

monolayers were infected at a multiplicity of infection of greater than 10 in DMEM containing 5 micrograms/ml trypsin. Cells were incubated with the virus for 48 hours or until a cytopathic effect was obtained. Media and cell debris were collected and centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant containing the rotavirus was then centrifuged at 10,000 x g in a preparative Beckman Ti45 rotor at 4°C. Virus pellets were resuspended in SM medium (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl<sub>2</sub>) and homogenized lightly in a Dounce-type homogenizer. The resuspended virus was centrifuged at 10,000 x g for 10 minutes then loaded onto 25-50% CsCl gradients in SM buffer. Gradients were centrifuged at 100,000 x g for 4 hours at 20°C. The two blue-white bands representing intact virions and cores of rotavirus were collected, diluted, and the CsCl gradient procedure was repeated a second time. Virus obtained from the second gradient was dialyzed overnight against SM buffer at 4°C.

Viral RNA Isolation. Dialyzed swine rotavirus was twice extracted with an equal volume of SDS/phenol then twice more with chloroform: isoamylalcohol (24:1). The double stranded RNA was precipitated with ethanol in the presence of 0.2 M sodium acetate, centrifuged and resuspended in water. The yield was typically 100 micrograms from 1,000 cm<sup>2</sup> of infected cells.

Synthesis and Cloning of gp38 cDNA. 160 micrograms of double-stranded swine rotavirus RNA obtained from the above procedure was mixed with one microgram each of two synthetic oligo nucleotide primers in a volume of 160 microliters (sequences of primers were:

5' CGGAATTCTCCAGGTACATACAAATTCTAATCTAAG-3' and  
5' CGAAATTCTCCAGGCTTAAAGAGAGAAATTCCGTTGGCTA-3')

derived from the published sequence of bovine rotavirus (24). The RNA-primer mixture was boiled for 3 minutes in a water bath then chilled on ice. Additions of 25 microliters of 1 M Tris-HCl pH 8.3, 35 micro-liters of 1 M KCl, 10 microliters of 0.25 M MgCl<sub>2</sub>, 7 microliters of 0.7 M 2-mercaptoethanol, 7 microliters of 20 mM dNTP's and 6 microliters of reverse transcriptase (100 units) were made sequentially. The reaction was incubated at 42°C for 1.5 hours then 10 microliters of 5 M EDTA pH 8.0 was added and the solution was extracted once with chloroform: phenol (1:1). The aqueous layer was removed and to it 250 microliters of 4 M ammonium acetate and 1.0 ml of 95% ethanol was added, the mixture was frozen in dry ice and centrifuged in the cold. The resulting pellet was resuspended in 100 microliters of 10 mM Tris-HCl pH 7.5 and the ammonium acetate precipitation procedure was repeated. The pellet was resuspended in 100 microliters of 0.3 M KOH and incubated at room temperature overnight then at 37°C for 2 hours. The solution was brought to neutral pH by addition of 10 microliters of 3.0 M HCl and 25 microliters of 1.0 M Tris-HCl pH 7.5. The resulting single-stranded cDNA was then precipitated two times by the above described ammonium acetate-ethanol procedure. The pellet obtained was resuspended in 50 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA boiled in a water bath for 2 minutes then incubated at 59°C for 16 hours. The solution was lyophilized to a volume of 15 microliters and the resulting double-stranded cDNA was run on a 1.0% agarose gel (Sigma agarose Type II). The ethidium bromide stained DNA migrating at 1,000-1,100 base pair length was excised from the gel and electroeluted in a CBS electroeluter device. The solution was lyophilized, and the cDNA was resuspended in 25 microliters of water. To this solution was added 2 microliters of 1.0 M Tris-HCl pH 7.5, 2 microliters of 1 M KCl, 1 microliter of 0.25 M MgCl<sub>2</sub>, 1 microliter of 20 mM dNTP's and 5 units of *E. coli* DNA polymerase I. The reaction was incubated at room temperature for 15 minutes, then chloroform/phenol extracted and ammonium acetate-ethanol precipitated as described above. The resulting cDNA was tailed with dCTP using terminal deoxynucleotide transferase (BRL buffer and enzyme used). The reaction was stopped with 2 microliters of 0.5 M EDTA, chloroform/phenol extracted and precipitated with sodium acetate in the presence of 10 micrograms of carrier tRNA. The resuspended cDNA was mixed with 200 ng of dGMP-tailed Pst I cut pER322 (BRL catalog #5355SA) in 200 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, heated to 65°C for 5 minutes then 57°C for 2 hours. The annealed cDNA-vector pBR322 was transformed onto *E. coli* DH-1 cells prepared for high efficiency transformation. Colonies that showed sensitivity to ampicillin and tetracycline resistance were grown and DNA was prepared and cut with Pst I to determine the size of the cDNA insert. Several clones having Pst I inserts of 1,050-1,100 base pairs were analyzed and found to have identical restriction enzyme digest patterns. The largest clone was designated pSY565 and has been deposited with the ATCC under accession number 53,340. For one of these clones, the 1,100 base pair Pst I insert was subcloned into a M13 phage sequencing vector. The entire DNA sequence of this clone was determined and is shown in Fig. 10A and 10B. The location of the gp38 open reading frame was determined from the amino acid homology to human and bovine sequences already published (44).

**METHOD FOR cDNA CLONING BOVINE ROTAVIRUS gp38 GENE Virus Growth.** The Calf Nebraska strain of bovine rotavirus (USDA) was propagated on MA-104 cells (Rhesus monkey kidney cells from MA Bioproducts). Confluent monolayers were infected at a multiplicity of infection of greater than 10 in DMEM containing 5 micrograms/ml trypsin. Cells were incubated with virus for 48 hours or until a cytopathic effect was obtained. Media and cell debris were collected and centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant containing the rotavirus was then centrifuged at 10,000 x g in a preparative Beckman Ti45 rotor at 4°C. Virus pellets were resuspended in SM medium (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl<sub>2</sub>) and homogenized lightly in a Dounce-type homogenizer. The resuspended virus was centrifuged at 10,000 x g for 10 minutes then loaded onto 25-50% CsCl gradients in SM buffer. Gradients were centrifuged at 100,000 x g for 4 hours at 20°C. The two blue-white bands representing intact virions and

cores of rotavirus were collected, diluted, and the CsCl gradient procedure was repeated a second time. Virus obtained from the second gradient was dialyzed overnight against SM buffer at 4°C.

5 **Viral RNA Isolation.** Dialyzed bovine rotavirus was twice extracted with an equal volume of SDS/phenol then twice more with chloroform: isoamylalcohol (24:1). The double stranded RNA was precipitated with ethanol in the presence of 0.2 M sodium acetate, centrifuged and resuspended in water. The yield was typically 100 micrograms from 1,000 cm<sup>2</sup> of infected cells.

**Synthesis and Cloning of gp38 cDNA.** 160 micrograms of double-stranded bovine rotavirus RNA obtained from the above procedure was mixed with one microgram each of two synthetic oligo nucleotide primers in a volume of 160 microliters (sequences of primers were:

**10** 5'-GGAAATTCTGCAGGTACATCATACAATTCTAATCTAAG-3' and  
5'-GGAAATTCTGCAGGCTTAAAAGAGAGAATTCCGTTGGCTA-3')

derived from the published sequence of bovine rotavirus (24). The RNA-primer mixture was boiled for 3 minutes in a water bath then chilled on ice. Additions of 25 microliters of 1 M Tris-HCl pH 8.3, 35 microliters of 1 M KCl, 10 microliters of 0.25 M MgCl<sub>2</sub>, 7 microliters of 0.7 M 2-mercaptoethanol, 7 microliters of 20 mM dNTP's, and 6 microliters of reverse transcriptase (100 units) were made sequentially. The reaction was incubated at 42°C for 1.5 hours than 10 microliters of 0.5 M EDTA pH 8.0 was added and the solution was extracted once with chloroform: phenol (1:1). The aqueous layer was removed and to it 250 microliters of 4 M ammonia acetate and 1.0 ml of 95% ethanol was added, the mixture was frozen in dry ice and centrifuged in the cold. The resulting pellet was resuspended in 100 microliters of 10 mM Tris-HCl pH 7.5 and the ammonium acetate precipitation procedure was repeated. The pellet was resuspended in 100 microliters of 0.3 M KOH and incubated at room temperature overnight then at 37°C for 2 hours. The solution was brought to neutral pH by addition of 10 microliters of 3.0 M HCl and 25 microliters of 1.0 M Tris-HCl pH 7.5. The resulting single-stranded cDNA was then precipitated two times by the above described ammonium acetate-ethanol procedure. The pellet obtained was resuspended in 50 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, boiled in a water bath for 2 minutes then incubated at 59°C for 16 hours. The solution was lyophilized to a volume of 15 microliters and the resulting double-stranded cDNA was run on a 1.0% agarose gel (Sigma agarose Type II). The ethidium bromide stained DNA migrating at 1,000-1,100 base pair length was excised from the gel and electroeluted in a CBS electroeluter device. The solution was lyophilized, and the cDNA was resuspended in 25 microliters of water. To this solution was added 2 microliters of 1.0 M Tris-HCl pH 7.5, 2 microliters of 1 M KCl, 1 microliter of 0.25 M MgCl<sub>2</sub>, 1 microliter of 20 mM dNTP's, and 5 units of *E. coli* DNA polymerase I. The reaction was incubated at room temperature for 15 minutes, then chloroform/phenol extracted and ammonium acetate-ethanol precipitated as described above. The resulting cDNA was tailed with dCTP using terminal deoxynucleotide transferase (BRL buffer and enzyme used). The reaction was stopped with 2 microliters of 0.5 M EDTA, chloroform/phenol extracted and precipitated with sodium acetate in the presence of 10 micrograms of carrier tRNA. The resuspended cDNA was mixed with 200 ng of dGMP-tailed Pst I cut pBR322 (BRL catalog #5355SA) in 200 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, heated to 65°C for 5 minutes then 57°C for 2 hours. The annealed cDNA-vector pBR322 was transformed onto *E. coli* DH-1 cells prepared for high efficiency transformation. Colonies that showed sensitivity to ampicillin and tetracycline resistance were grown and DNA was prepared and cut with Pst I to determine the size of the cDNA insert. Several clones having Pst I inserts of 1,050-1,100 base pairs were analyzed and found to have identical restriction enzyme digest patterns. For one of these clones, the 1,100 base pair Pst I insert was subcloned into a M13 phage sequencing vector. Part of the DNA sequence of this clone was determined and was found to be identical to the published sequence (24).

**SELECTION OF G418 RESISTANT HERPESVIRUS.** The antibiotic G418 (GIBCO) has a wide range of inhibitory activity on protein synthesis. The recombinant virus, however, expressed the aminoglycoside 3'-phosphotransferase, encoded by the NEO gene, upon acquiring the foreign gene and became resistant to G418. The transfection stocks of recombinant viruses were grown on MDBK (for IBR virus), Vero (for PRV) or QT35 (for HVT) cells in the presence of 500 micrograms/ml G418 in complete DME medium plus 1% fetal bovine serum. After one or two days at 37°C, plaques from the dishes inoculated with the highest dilution of virus were picked for virus stocks. The selection was repeated a second or third time. The virus stocks generated from the G418 selection were tested for NEO gene insertion by the SOUTHERN BLOTTING OF DNA hybridization procedure described above.

**50** **PURIFICATION OF gpX.** gpX was purified from the tissue culture medium of infected Vero cells grown in complete DME plus 1% fetal bovine serum. Confluent Vero cells were infected at a multiplicity of infection equal to 5, with wild-type, Iowa S-62 strain pseudorabies virus. The viral proteins were radiolabelled with <sup>14</sup>C glucosamine and/or <sup>35</sup>S methionine by adding the appropriate label to the flask eight hours after infection. The cells and media were harvested at twenty hours post infection, when the cells showed considerable cytopathic effect and the fluids were centrifuged.

**55** The supernatant fluid was concentrated 10X and dialyzed against 0.02M sodium sulfate/0.01M sodium phosphate buffer, pH 7.2 (16 hours, 0°C), then against two changes of 0.01M sodium phosphate buffer, pH 7.2 (24 hours, 0°C). The dialysate was treated for 30 minutes at 0°C with 70% perchloric acid to a final concentration of 0.2M perchloric acid, then centrifuged at 10,000 rpm for 25 minutes. The supernatant fluid was then dialyzed against 0.02M Tris, pH 8.5.

Purification was carried out by high performance liquid chromatography on a Beckman Model 334 HPLC.

The acid-soluble proteins were separated on a Biogel TSK DEAE 5-PW column (75 x 75mm) using a 60 minute linear gradient, flow rate 0.8 ml/minute. Starting buffer was 0.02M Tris, pH 8.5, limit buffer was 0.02M Tris, pH 7.0 containing 0.75M NaCl.

5 The gpX eluted as a major radioactive peak at 64% of the limit buffer. The recovered material represented 25% of the applied radioactivity.

ELISA ASSAY. A standard enzyme-linked immunosorbent assay (ELISA) protocol was used to determine the immune status of swine following vaccination and challenge.

A purified gpX antigen solution (40 microliters) was allowed to absorb to the wells of polycarbonate microtiter dishes for 2 hours at room temperature. The antigen was in a (0.015M) carbonate-(0.04M) bicarbonate buffer, pH 9.6.

10 The coated wells were rinsed 3 times with ELISA wash solution (0.05% Tween 20 non-ionic detergent in phosphate buffered saline, pH 7.5).

Forty microliters of serum containing gpX antibody (diluted 1 to 10 in Tris buffer containing 1% bovine serum albumin and 0.05% Tween 20) were added to the wells and incubated 1 hour at 37°C.

15 The anti-serum was removed and the wells were washed 3 times with ELISA wash solution. A solution containing Staphylococcal protein A coupled to horseradish peroxidase (Bio-Rad) (diluted 1:10,000 in the Tris/BSA/Tween buffer described above) was added (50 microliters) to visualize the wells containing antibody against the specific antigen. The solution was incubated 1 hour at 37°C, then removed and the wells were washed 3 times with ELISA wash solution.

100 microliters of substrate solution (equal volumes of hydrogen peroxide and ATBS buffer (Bio-Rad) were added to each well and color as allowed to develop for 20 minutes.

20 The reaction was terminated by addition of 50 microliters of 0.01M oxalic acid. The color was read at absorbance (A) 410nm on a automatic plate reader.

VACCINATION STUDIES IN SWINE. Weaned pigs (4-6 weeks old) and pregnant sows were obtained from swine herds known to be free of pseudorabies disease. Susceptibility of the test animals to pseudorabies was further verified by testing the pig serum for absence of neutralizing antibodies to pseudorabies virus (PRV). The weaned pigs and 3-

25 to-4 day old piglets were inoculated intramuscularly with 1 ml of virus fluid containing about  $10^4$  to  $10^6$  infectious units (TCID<sub>50</sub>). Animals were observed each day after vaccination for adverse reactions (clinical signs of PRV disease) and body temperatures were recorded. Samples of tonsillar secretions were obtained and cultured to determined if the vaccine virus was capable of shedding and spreading to other animals. Immunity was determined by measuring PRV

30 serum antibody levels at weekly intervals and in some cases, by challenging the vaccinated pigs with virulent virus. In the latter case, the vaccinated animals and a group of non-vaccinated pigs were inoculated with virulent, Iowa S-62 strain PRV, using an amount of virus that caused PRV disease in at least 80% of the unvaccinated group of pigs. This was done about 28 days after vaccination. The challenged animals were observed daily for signs of disease and for increased body temperatures. A necropsy was conducted on animals that died and selected tissues were examined and cultured for PRV.

35 **EXAMPLES**

**EXAMPLE 1**

40 **S-PRV-004**

We have created a virus that has a deletion in the junction region between the unique long DNA and the internal repeat of PRV, and a deletion in the endogenous PRV thymidine kinase gene in the unique long region. Into the junction deletion we have cloned the herpes simplex type 1 (HSV-1) thymidine kinase (TK) gene under the control of the ICP4 promoter. This virus is designated S-PRV-004.

45 To create this virus, we first cloned the Sall #1 fragment of PRV. PRV DNA was prepared and then cut with Sall restriction enzyme. The cut DNA was electrophoresed on an agarose gel and the largest Sall band (15 kb) was purified from the gel (see PHENOL EXTRACTION OF DNA FROM AGAROSE). The purified DNA was ligated into the plasmid pSP64 (see LIGATION) and the DNA mixture was used to transform *E. coli* HB101 according to Maniatis et al. (1). The Sall #1 clone was mapped for restriction sites.

50 The homologous recombination procedure was used to create S-PRV-004 (see Fig. 2). The exact position of the junction region was determined by sequencing the DNA from Sall #1 fragment. It was found that the junction region was positioned between two StuI sites (Fig. 2A). Two fragments of DNA from the Sall clone were used to create the homology vector for recombination. One was a fragment from BamHI #8' from StuI to BamHI and the other was from BamHI #8 from BamHI to StuI (see Figs. 1B and 2A). These fragments were cloned into the BamHI site of pSP64. This plasmid was cut with StuI, and a 3.8 kb Pvull fragment, obtained from B. Roizman (16), The University of Chicago, and containing the ICP4 promoter on the BamHI-N fragment and the HSV-1 TK gene on the BamHI-Q fragment, fused at the BamHI/BgIII sites, was ligated into the StuI site. The net result from this series of clonings was a plasmid which had suffered a deletion of 3kb from between the StuI sites, and into which 3.8kb of the foreign TK gene had been incorporated

(see Fig. 2B). The TK gene was thus flanked by PRV DNA sequences to allow for insertion of the foreign gene into the PRV genome by homologous recombination. The plasmid DNA was transfected into rabbit skin cells along with the intact PRV DNA from S-PRV-003, which is a pseudorabies virus that has a deletion in the endogenous TK gene. The transfection stock of virus was selected in HAT medium and the virus was identified and selected by analysis of the restriction pattern of DNA isolated from the infected cells.

S-PRV-004 contained the HSV-1 TK gene and was expressing this gene as demonstrated by the incorporation of <sup>14</sup>C-thymidine in a plaque assay described in Tenser et al. (40) and by direct analysis of TK activity in infected cell extracts, following the procedure of Cheng et al. (41). The location of this gene in the genome of PRV is shown in Figure 2C.

Six weaning age pigs were vaccinated with  $10^{5.0}$  infectious units of S-PRV-004 and challenged with virulent PRV 28 days later, according to the VACCINATION STUDIES IN SWINE procedure. The vaccinated pigs remained healthy following vaccination and developed serum neutralizing antibody against PRV (see Table I below). Vaccine virus was not recovered from nasal or tonsillar secretions. After exposure to virulent PRV, 83% of the vaccinated swine were protected against PRV disease.

TABLE I  
RESPONSES OF WEANED PIGS VACCINATED WITH S-PRV-004 AND CHALLENGED WITH VIRULENT PRV

Anti- gen Level	Pig No.	Post-Vaccination					Post-Challenge			
		Antibody			Clinical Signs	Virus Isolation	Antibody		Clinical Signs <sup>a</sup>	Virus Isolation
		Day 14	Day 21	Day 28			Day 7	Day 14		
$10^{5.0}$	1	32	32	16	None	None	>64	>64	F	Swabs
	2	16	32	8	None	None	>64	>64	F	Swabs
	3	8	16	4	None	None	>64	>64	F	Swabs
	4	4	16	8	None	None	>64	>64	F,C	Swabs
	5	16	16	8	None	None	>64	>64	F	Swabs
	6	8	8	4	None	None	>64	>64	F	Swabs

<sup>a</sup>Key to clinical signs: C=CNS, F=Febrile

#### EXAMPLE 2

##### S-PRV-005

S-PRV-005 is a pseudorabies virus that has a deletion in the repeat region and in the endogenous PRV TK gene in the long unique region, and has an insertion of the HSV-1 TK gene under the control of the ICP4 promoter incorporated into both copies of the repeat region between the XbaI site and the HpaI site in the BamHI #5 fragment (See Fig. 3).

To create this virus, we first obtained a clone of BamHI #5 fragment from PRV (Fig. 1B). The BamHI #5 fragment was cloned into the plasmid pACYC184 at the BamHI site (see LIGATION above). A map of the BamHI #5 fragment is shown in Fig. 3A.

The plasmid containing the BamHI #5 fragment was cut with XbaI and HpaI and the linearized plasmid was purified (see PHENOL EXTRACTION OF DNA FROM AGAROSE). The 3.8kb Pvull fragment described in Example 1 and containing the TK gene and ICP4 promoter was likewise purified. The XbaI site was filled to yield a blunt end (see POLYMERASE FILL-IN REACTION), and the two DNAs were mixed and ligated together. The resulting plasmid that had incorporated the TK gene in the XbaI-HpaI deletion was selected and analyzed by restriction mapping (Fig. 3B).

The plasmid containing the TK gene flanked by PRV Bam HI #5 sequences was used to transfect rabbit skin cells along with purified DNA from S-PRV-003, a pseudorabies virus that had a deletion in the endogenous TK gene. The resulting recombinant PRV that had incorporated the HSV-1 TK gene into the deletion in the repeats was screened and purified from the transfection stock by the HYBRIDIZATION SCREEN FOR RECOMBINANT HERPESVIRUS proce-

dure without any prior selection.

S-PRV-005 recombinant PRV was shown to express the HSV-1 TK gene by incorporation of  $^{14}\text{C}$ -thymidine in a plaque assay (40), by analysis of the TK activity in infected cell lysates (41), and by immunodetection of the HSV-1 TK protein according to the ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure outlined above. The location of this gene in the genome of PRV is shown in Fig. 3C.

EXAMPLE 3

S-PRV-010

S-PRV-010 is a pseudorabies virus that has a deletion in the PRV TK gene in the long unique region, a deletion in the repeat region, and the insertion of the *E. coli* beta-galactosidase gene (lacZ gene) incorporated into both copies of the repeats at the XbaI site in BamHI #5 fragment (see Fig. 5A). The beta-galactosidase gene was constructed to be expressed using the HSV-1 TK gene promoter which we have shown in this construct to be active in PRV.

The method used to insert the beta-galactosidase gene into S-PRV-010 was direct ligation (see DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS). The beta-galactosidase gene was on plasmid PJF751, obtained from Jim Hoch, Scripps Clinic and Research Foundation. This gene is truncated at the 5' end with a BamHI site that has removed the AGT initiation codon, and the Aval site in pBR322 was used at the other end (see Fig. 4A). The HSV-1 TK promoter (Fig. 4B) was taken from the McKnight TK gene as an RsaI fragment, gel purified, and ligated to a synthetic piece of DNA which contained a BamHI site within the sequence CGGATCCG (Fig. 4C). After digestion with BamHI, the fragment was cloned into the BamHI site at the start of the beta-galactosidase gene (Fig. 4D). The plasmid was constructed with the *E. coli* plasmids pSP64 and pSP65 such that XbaI sites from the polylinkers could be used to excise the entire construct from the plasmid. The ligation mixture was used to transfect *E. coli* HB101 according to published procedures (Maniatis et al. (1)). This construct was planned such that the first three amino acids of the protein were from the HSV-1 TK gene, the next three were from the synthetic linker, and the rest were from the beta-galactosidase gene. The gene contained the following sequence at the fusion between TK and lacZ:

32 5' CGT ATG GCT TCG TCG GAT CCC GTC GTT TTA.....3'  
 MET ala ser ser asp pro val val leu.....  
 -----  
 35 TK gene ----- lac Z  
 -----  
 BamHI linker

40 A pseudorabies virus construct designated S-PRV-002 which has a deletion in the PRV TK gene in the unique long region and a deletion in the repeat region was used as the recipient for the beta-galactosidase gene. Intact S-PRV-002 DNA was mixed with a 30-fold molar excess of plasmid DNA containing the beta-galactosidase gene under the control of the HSV-1 TK promoter, and this mixture was digested with XbaI restriction enzyme. The ligated DNA was used to transfect animal cells, and the transfection stock was analyzed for recombinant PRV. First, PRV DNA was prepared from 45 cells infected with the transfection stock virus and this DNA was cut with restriction enzymes and analyzed on an agarose gel. This analysis showed that the recombinant virus was present as the major species in the transfection stock, and it was subsequently purified from other virus species by plaque assay coupled with the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS. Because beta-galactosidase reacted with the drug Bluogal™ to yield a product with blue color, it was possible to plaque purify the recombinant by picking blue plaques.

50 The final result of the purification was the recombinant PRV designated S-PRV-010. It was shown to express the enzyme beta-galactosidase by the formation of blue plaques as noted above, and by the detection of the enzyme in infected cell extracts using the substrate O-nitrophenyl-beta-D-galactopyranoside (Sigma) following the procedure of Norton and Coffin (33). The location of this gene in the genome of PRV is shown in Fig. 5C.

55 Previous studies demonstrated that swine vaccinated with S-PRV-002 developed antibody to PRV and were fully protected against clinical disease following exposure to virulent PRV virus. Animal studies were conducted with S-PRV-010 to determine the utility of a recombinant pseudorabies virus as a vaccine against pseudorabies disease.

A group of weaned pigs and a litter of four-day-old piglets were vaccinated with S-PRV-010 and challenged three to four weeks later, according to VACCINATION STUDIES IN SWINE.

Responses of weaned pigs vaccinated with S-PRV-010 are shown in Table II. Administration of this virus did not

cause adverse reactions in the pigs. The vaccinated animals developed PRV neutralizing antibody. Two, non-vaccinated control animals (#75 and #91) placed in contact with the vaccines did not develop PRV antibody prior to challenge, indicating the vaccine virus was not shed from vaccines. After challenge, all ten vaccinated animals remained clinically normal and free of PRV disease. In contrast, the two in-contact control animals and three of five non-vaccinated control animals developed PRV disease and one of these pigs died of PRV.

To test further the utility of S-PRV-010 as a vaccine, the virus was inoculated into 4-day old piglets. The results, presented in Table III, demonstrated that the virus elicited an antibody response in vaccinated piglets and did not cause adverse reactions. The virus apparently was shed from vaccines, since one (#67) of two non-vaccinated, in-contact control piglets had developed PRV antibody by Day 24. After challenge, all vaccinated animals and the sero-positive in-contact control animal remained free of PRV disease. By comparison, the three non-vaccinated control pigs and the second in-contact control pig developed clinical signs of PRV and died.

The conclusion from that study is that S-PRV-010 given at a dosage of  $10^{4.0}$  or  $10^{6.0}$ , elicits a protective response in vaccinated piglets or weaned pigs capable of preventing infection by virulent virus.

15 TABLE II  
20 SEROLOGIC AND CLINICAL RESPONSES OF WEANED PIGS FOLLOWING VACCINATION WITH S-PRV-010  
25 AND CHALLENGE WITH WILD-TYPE PRV

Vaccine GROUP	Antibody Titers <sup>a</sup>						Post-Challenge Clinical Signs
	Pig Number	Post-Vaccination			Post-Challenge		
		Day 0	Day 14	Day 24	Day 7	Day 14	
$10^{6.0}$ Per Dose	70	<2	64	32	32	64	None
	71	<2	16	16	16	32	None
	72	<2	64	32	16	64	None
	73	<2	64	16	16	64	None
	74	<2	16	8	4	4	None
	75 <sup>b</sup>	<2	<2	<2	<2	4	Depressed, Dyspnea, CNS Signs <sup>c</sup>
$10^{4.0}$ Per Dose	76	<2	64	4	8	32	None
	77	<2	16	16	64	8	None
	78	<2	32	16	32	8	None
	79	<2	8	16	64	4	None
	80	<2	2	<2	256	16	None
	81 <sup>b</sup>	<2	<2	<2	<2	16	Depressed, Rhinitis, CNS Signs
Controls	82	NT	NT	<2	<2	8	None
	83	NT	NT	<2	<2	16	None
	84	NT	NT	<2	<2	32	CNS Signs, Depressed, Dyspnea
	85	NT	NT	<2	<2	64	CNS Signs
	86	NT	NT	<2	<2	-	CNS Signs Died
	NT: Not Tested						

55 <sup>a</sup> Determined by RIDEA

<sup>b</sup> In-contact Controls

<sup>c</sup> CNS signs include Ataxia, Incoordination, Circling, Lateral Recumbency

TABLE III

SEROLOGIC AND CLINICAL RESPONSES OF 4-DAY-OLD PIGLETS FOLLOWING VACCINATION WITH S-PRV-010 AND CHALLENGE WITH WILD-TYPE PRV							
Vaccine GROUP	Pig Number	Antibody Titers <sup>a</sup>				Post-Challenge Clinical Signs	
		Post-Vaccination			Post-Challenge		
$10^{6.0}$ Per Dose	60	<2	4	16	16	32	None
	61	<2	64	8	64	8	None
	62	<2	32	2	16	16	None
$10^{4.0}$ Per Dose	63	<2	- <sup>b</sup>	-	-	-	-
	64	<2	64	2	32	16	None
	65	<2	2	4	32	16	None
In-Contact Controls	66	<2	2	NT	- <sup>c</sup>	-	Comatose, Died
	67	<2	<2	8	64	32	None
	87	NT	NT	<2	- <sup>c</sup>	-	CNS Signs <sup>d</sup> , Died
	88	NT	NT	<2	- <sup>c</sup>	-	CNS Signs, Died
	89	NT	NT	<2	- <sup>c</sup>	-	Died
	NT: Not Tested						

<sup>a</sup> Determined by RIDEA<sup>b</sup> Died 8 Days Post Vaccination From Ruptured Stomach<sup>c</sup> Died on or Prior to Day 7 Post-Challenge<sup>d</sup> CNS Signs include Ataxia, Incoordination, Circling Lateral RecumbencyEXAMPLE 4S-PRV-007

S-PRV-007 is a pseudorabies virus that has a deletion in the PRV TK gene in the unique long region, a deletion in the repeat region, and the swine rotavirus glycoprotein 38 gene under the control of the HSV-1 ICP4 promoter inserted into the repeat region.

S-PRV-005 virus described in Example 2 above was further engineered to contain the rotavirus antigen (see Fig. 6) as follows. The swine rotavirus gp38 gene was cloned into plasmid pBR322 at the PstI site by procedures previously described herein. The resulting plasmid was called pSY565 (see Fig. 7). The 1090 bp PstI fragment containing the gp38 gene was cloned into vector pUC4K at the PstI site such that it became flanked by BamHI sites in a plasmid called pSY762.

Plasmid pSY590 has has a complex origin as inferred from the flow chart. These clonings were routine in nature and are of historical interest but are not strictly required to practice the invention. Briefly this history is:

(1) The McKnight TK gene was the HSV-1 BamHI Q fragment from HaeIII at -178 relative to CAP site to BamHI at +2700 which was cloned between HindIII and BamHI in pBR327.

(2) pSY491..The entire TK coding region from the BglII site at +55 (relative to CAP site) to the BamHI site at +2700

was cloned into the BamHI site in pSP65 and called pSY491.

5 (3) pSY481..The polyA signal sequence (pA) on an 800 bp SmaI fragment from TK was subcloned into the SmaI site in pSP65 and was called pSY481.

10 (4) pSY583..The pA 800 bp SmaI fragment from pSY481 was cloned into the HinclI site in pSP65 and called pSY583.

15 (5) pSY429..The HSV-1 BamHI N fragment was obtained from Dr. B. Roizman cloned into the BamHI site of pBR322 and was called pSY429.

20 (6) pSY584..The 2.2kb fragment of BamHI N from Pvull to BamHI (Ref. 16) in pSY420 was subcloned into pSP65 between HinclI and BamHI in the polylinker and was called pSY584.

25 (7) pSY479..A plasmid was constructed from pSP64 and pSP65 that contained a fused polylinker sequence. Both plasmids were cut with PstI in the polylinker and Pvul in the plasmid body. The net effect of this construct was to create a fusion plasmid called pSP66 which has a symmetrical polylinker sequence centered on the PstI site. pSY479 is the name of this plasmid and it also contained a PstI fragment cloned into the PstI site that is irrelevant for the manipulations that follow.

30 Plasmid pSY590 was created from pSY583, pSY584, and pSY479 in a three fragment ligation of the following elements: the 3kb plasmid sequences from pSY479 (pSP66) cut with PstI, the 800 bp SmaI pA fragment cut from the polylinker in pSY583 with PstI and BamHI, and the 2200 bp BamHI N fragment cut from pSY583 with PstI and BamHI. Fig. 7 shows the final configuration of all of these DNA fragments in pSY590. There is a single BamHI site in the plasmid between the promoter in BamHI N and the TK pA signal that was used to insert the coding region of the gp38 gene.

35 For the creation of the homology vector used in the formation of S-PRV-007, the plasmid pSY590 was opened with BamHI, and the 1090 bp gp38 gene was removed from pSY762 by cutting with BamHI, and these two fragments were ligated together to form pSY596. The correct orientation of the gp38 gene was confirmed by diagnostic restriction enzyme digestion utilizing sites with gp38 (see Fig. 10A and 10B).

40 In pSY596 described above, the gp38 gene resided between two flanking HSV-1 DNA fragments. These two regions were thus homologous to similar regions on the HSV-1 TK gene in S-PRV-005, and these regions were used for the homologous recombination to create S-PRV-007 (Fig. 6A). The plasmid and S-PRV-005 DNAs were mixed and used in the DNA TRANSFECTION PROCEDURE FOR GENERATING RECOMBINANT VIRUS. A virus that had incorporated the rotavirus antigen in place of the TK gene was selected with BUDR. Recombinants from the selected virus stock that had incorporated the rotavirus DNA were screened by the HYBRIDIZATION SCREEN FOR RECOMBINANT HERPESVIRUS and by analyzing restriction digests of DNA by the SOUTHERN BLOTTING OF DNA procedure using the rotavirus cloned gp38 gene as probe.

45 The final result of this screening was a recombinant PRV called S-PRV-007 which had the rotavirus gp38 gene incorporated into the repeat region between the XbaI and HpaI sites in PRV BamHI #5 fragment shown in Figure 6C. The presence in a host of gp38 expressed by S-PRV-007 has not yet been detected.

#### EXAMPLE 5

##### S-PRV-012

50 S-PRV-012 is a pseudorabies virus that has a deletion in the PRV TK region in the unique long region, a deletion in the repeat region, and a deletion in the unique short region encoding the PRV glycoprotein X, called gpX and identified and mapped by Rea et al. (23). The HSV-1 TK gene under the control of the ICP4 promoter was inserted in place of the gpX gene.

55 The following procedure was used to make the deletion of gpX and the simultaneous insertion of the HSV-1 TK gene. The flanking regions for homology to PRV were from cloned fragments of BamHI #10 fragment and BamHI #7 fragment extending from NdeI to BamHI (Fig. 8). The BamHI and NdeI sites were filled in according to the POLYMER-ASE FILL-IN REACTION, and the Pvull fragment of HSV-1 DNA was inserted by LIGATION. This plasmid was transfected with intact S-PRV-002 DNA according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. The recombinant virus was selected by HAT SELECTION OF RECOMBINANT HERPESVIRUS procedure, and screened by the ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure using antibodies specific for the HSV-1 protein.

The recombinant virus selected by this procedure was designated S-PRV-012 and has been deposited with the ATCC under Accession No. VR-2119 and was shown by RESTRICTION MAPPING OF DNA and SOUTHERN BLOTTING

TING OF DNA to contain the HSV-1 TK gene inserted in place of the gpX gene (Fig. 8B). The ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure showed that the virus was expressing the inserted HSV-1 TK gene. The structure of this virus is shown in Figure 8C.

5 EXAMPLE 6

S-PRV-013

10 S-PRV-013 is a pseduorabies virus that has a deletion in the TK gene in the long unique region, a deletion in the repeat region and a deletion in the gpX coding region. The gene for E. coli beta-galactosidase (lacZ gene) was inserted in place of the gpX gene and is under the control of the endogenous gpX gene promoter.

15 The following procedures were used to construct S-PRV-013 by homologous recombination. The flanking PRV homology regions were from the cloned BamHI #10 fragment which contained the gpX promoter, and from the cloned BamHI #7 fragment extending from the NdeI site to the BamHI site (Figure 9A). The NdeI site was filled in according to the POLYMERASE FILL-IN REACTION, and the beta-galactosidase gene was inserted between the BamHI #10 and BamHI #7 fragments. This construct positioned the beta-galactosidase gene between the gpX promoter and the gpX poly A signal sequences with a deletion of almost all of the coding regions of gpX. The plasmid DNA and DNA from S-PRV-002, a PRV strain with a deletion in both repeat sequences and a deletion in the thymidine kinase gene, were mixed and transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. The recombinant virus was screened and purified from the transfection stock by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure.

20 The resulting virus from this screen was designated S-PRV-013 and has been deposited with the ATCC under Accession No. VR 2120. It contained the beta-galactosidase gene in place of the gpX coding regions (Figures 9B and 9C) as determined by PREPARATION OF HERPESVIRUS DNA followed by SOUTHERN BLOTTING OF DNA. The expression of the beta-galactosidase gene was confirmed by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS test, and by the o-nitrophenylgalactopyranoside substrate assay (33).

25 To confirm that the coding region for gpX had been removed from S-PRV-013, DNA extracted from a stock of purified S-PRV-013 was digested with BamHI and the fragments were separated on agarose gel electrophoresis and analyzed by SOUTHERN BLOT HYBRIDIZATION. The hybridization probe was the BamHI-NDE fragment of pseudorabies 30 BamHI #7 fragment from the unique short region. This probe fragment included 90% of the coding sequences of gpX. In this analysis, the gpX region was shown to be missing from S-PRV-013.

35 To confirm these results, cells were infected with either wild-type pseudorabies, S-PRV-012 or S-PRV-013, and samples of media from the infected cultures were subjected to SDS-polyacrylamide gel electrophoresis. The cell was blotted and analyzed using the WESTERN BLOTTING PROCEDURE. The anti-serum used was a rabbit hyper-immune serum raised against a chemically-synthesized gpX antigenic peptide linked to bovine serum albumin. As shown in Figure 4, gpX is prominent in the media of cells infected with wild-type virus (PRV 000), but is not detected in the media of cells infected with S-PRV-012 or S-PRV-013. These results demonstrate that the pgX gene is missing from both S-PRV-012 and S-PRV-013 and the protein, gpX, is not produced in cells infected by either S-PRV-012 or S-PRV-013.

40 The following experiments indicate that S-PRV-013 may be used as a vaccine to protect swine against psuedorabies disease and that it produces an immune response which can readily be distinguished from wild-type infection.

45 In the first study, susceptible weaned pigs and four-day old piglets were vaccinated intramuscularly with S-PRV-013 as follows: 4 of each group were inoculated with  $10^6$  TCID<sub>50</sub> and 4 were inoculated with  $10^4$  TCID<sub>50</sub> of virus. The animals were observed, then challenged as described in VACCINATION STUDIES WITH SWINE (see Table IV below).

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TABLE IV  
RESPONSES OF 4-DAY-OLD PIGLETS VACCINATED  
WITH S-PRV-013 AND CHALLENGED WITH VIRULENT PRV

Pig Group	Vaccine Dose	Pig No.	Post-Vaccination				Post-Challenge			
			Antibody Day 14	Antibody Day 21	Clinical Signs Day 28	Virus Isolation	Antibody Day 7	Antibody Day 14	Clinical Signs	
WEANED	$10^6$ TCID <sub>50</sub>	1	4	2	2	NEG	NT <sup>b</sup>	>64	>64	NEG
		2	4	2	2	NEG	NT	>64	>64	NEG
		3	2	2	2	NEG	NT	64	>64	NEG
		4	4	2	4	NEG	NT	>64	>64	NEG
	$10^4$ TCID <sub>50</sub>	5	2	2	2	NEG	NT	>64	>64	NEG
		6	<2	<2	2	NEG	NT	64	>64	NEG
		7	<2	<2	<2	NEG	NT	64	>64	NEG
		8	<2	<2	<2	NEG	NT	64	>64	NEG
PIGLETS	$10^6$ TCID <sub>50</sub>	10	8	16	32	NEG	NEG	>64	>64	NEG
		11	- <sup>c</sup>	-	-	NEG	NEG	-	-	-
		12	8	NT	64	NEG	NEG	>64	>64	NEG
		13	4	8	32	NEG	NEG	>64	>64	NEG
	$10^4$ TCID <sub>50</sub>	14	4	8	32	NEG	NEG	>64	>64	NEG
		15	8	16	32	NEG	NEG	>64	>64	S
		16	2	2	8	NEG	NEG	64	>64	NEG
		17	4	16	32	NEG	NEG	64	64	NEG
	Contact Control	18	<2	<2	<2	NEG	NEG	2	16	F,C
		19	- <sup>d</sup>	-	-	NEG	NEG	-	-	-
Challenge Control		20					<2	-	F,C,D	
		21	Not Applicable				<2	2	F,C	
		22					<2	2	F,C	

Key to clinical signs: NEG=Negative,  
C=Conus, D=Death, F=Febrile, R=Respiratory,

S=Scours

<sup>b</sup>Not tested

<sup>c</sup>Sacrificed Day 4 post-vaccination

<sup>d</sup>Sacrificed Day 7 post-vaccination;  
runt doing poorly

Following vaccination, all animals were free of adverse reactions and all but 2 (weaned pigs) developed serum neutralizing antibody titers of 1:2 to 1:64. Virus was not recovered from tonsillar swabs of any pig or from tissues taken from the piglet (#11) sacrificed on Day 4. One of 2 contact control piglets (#19) was sacrificed 7 days into the experiment because it was a runt and doing poorly. Tissues from this piglet were negative when cultured for PRV. The other contact

**EP 0 794 257 A1**

control remained healthy and did not develop PRV antibody prior to challenge.

After challenge, all vaccinated animals remained clinically normal and developed secondary antibody responses. The contact control piglet and the three challenge control pigs all developed typical central nervous system signs of PRV and one control died following challenge.

5 In a second study with S-PRV-013 using larger numbers of animals, 2 litters of susceptible 3-day-old piglets and a group of 15 susceptible weaned pigs were vaccinated with  $10^4$  TCID<sub>50</sub> of virus, then challenged as described in VACCINATION STUDIES WITH SWINE (see Tables V and VI below).

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**Table V**  
**RESPONSES OF 3-DAY-OLD PIGLETS VACCINATED WITH**  
**S-PRV-013 AND CHALLENGED WITH VIRULENT PRV**

Group	Pig No.	Post-Vaccination				Post-Challenge			
		Antibody Day 7	Antibody Day 14	Clinical Signs <sup>a</sup>	Virus Isolation	Antibody Day 7	Antibody Day 14	Clinical Signs	Virus Isolation
LITTER A VACCINATES	1	<2	2	4	4	F <sup>b</sup>	Neg	32 >64	Neg
	2	<2	8	8	16	F	Neg	64 >64	Neg
	3	<2	8	8	16	F	Neg	16 32	Neg
	4	<2	8	16	16	F	Neg	32 >64	Neg
	6	<2	8	8	16	F	Neg	64 >64	Neg
	7	<2	<2	<2	<2	Neg	Neg	2 2	C,F,R Neg
LITTER B VACCINATES	8	<2	<2	<2	<2	Neg	Neg	<2 >64	C,F Neg
	10	<2	8	8	16	F	Neg	16 >64	Neg
	11	<2	8	8	16	F	Neg	32 >64	Neg
	12	<2	8	32	32	F	Neg	32 >64	Neg
	13	<2	4	16	32	F	Neg	64 >64	Neg
	14	<2	8	16	32	Neg	Neg	64 >64	Neg
CHALLENGE CONTROLS	16	<2	4	4	16	F	Neg	32 >64	Neg
	17	<2	8	8	32	F	Neg	64 >64	Neg
	18	<2	<2	<2	<2	Neg	Neg	2 2	C,F Neg
	19		<2					<2 2	C,F,R Neg
	20		<2					<2 2	C,F,R Sub
	21	Not Applicable	<2	Not Applicable				2 <2	C,F,R Sub
	22		<2					<2 <2	C,F,R Sub
	23		<2					<2 Died	C,D,F, R Sub Infil, QNS
	24		<2					<2 <2	C,F,R Sub

<sup>a</sup>Clinical signs: NEG = Negative, C=QNS, D=Death, F=Febrile, R=Respiratory  
<sup>b</sup>A 1°F increase in temperature was observed in day 1 in these vaccinates

TABLE VI

RESPONSE OF WEANED PIGS VACCINATED WITH  
S-PRV-013 AND CHALLENGED WITH VIRULENT PRV

Group	Pig No.	Post-Vaccination						Post-Challenge						
		Antibody Day 0	Antibody Day 14	Antibody Day 21	Clinical Signs	Virus Isolation	Antibody Day 7	Antibody Day 14	Clinical Signs	Virus Isolation	Antibody Day 7	Antibody Day 14	Clinical Signs	Virus Isolation
VACCINATES	35	<2	<2	4	Neg	Neg	>64	>64	Neg	Neg				
	36	<2	2	2	Neg	Neg	>64	>64	Neg	Neg				
	37	<2	2	2	Neg	Neg	>64	>64	Neg	Neg				
	38	<2	<2	2	Neg	Neg	>64	>64	Neg	Neg				
	39	<2	2	2	Neg	Neg	64	64	Neg	Neg				
	40	<2	2	4	Neg	Neg	>64	>64	Neg	Neg				
	41	<2	2	4	Neg	Neg	64	>64	Neg	Neg				
	42	<2	2	4	Neg	Neg	>64	>64	P	Neg				
	43	<2	2	2	Neg	Neg	>64	>64	P	Neg				
	44	<2	2	2	Neg	Neg	64	>64	P	Neg				
	45	<2	2	4	Neg	Neg	>64	>64	Neg	Neg				
	46	<2	2	2	Neg	Neg	>64	>64	Neg	Neg				
CONTROLS	30	<2	NT <sup>b</sup>	<2	Not Applicable			<2	4	C,F,R	Neg			
	31	<2	NT	<2				<2	2	C,F	Neg			
	32	<2	NT	<2				<2	4	C,F,R	Neg			
	33	<2	NT	<2				<2	Died	C,D,F,R	Terminal			
	34	<2	NT	<2				<2	4	P	Q5			

<sup>a</sup>Clinical signs: NEG=Negative, C= CNS, D=Death, F=Febrile, R=Respiratory<sup>b</sup>Not tested

In this experiment, all of the vaccinated animals remained healthy following vaccination, developed serum neutralizing antibody to PRV and did not shed vaccine virus in tonsillar secretions. After challenge with virulent virus, vaccines of both age groups remained free of PRV disease, whereas the 3 non-vaccinated contact controls and 10 of 11

of the challenge controls developed severe pseudorabies disease.

The serum samples collected from the vaccinated and challenged swine were assayed by the gpX ELISA assay. Because the gene for gpX was deleted from S-PRV-013, it is expected that swine vaccinated with S-PRV-013 would be sero-negative in the ELISA test for this antigen. The challenge virus carries the gpX gene. The vaccinated animals were 5 protected by the vaccination from pseudorabies disease when challenged with the wild-type virus. However, vaccinated animals were asymptotically super-infected by the challenge strain and would, therefore, be expected to produce antibodies to gpX upon challenge.

As shown in Figure 5, serum from an animal vaccinated with S-PRV-013 remained negative for gpX until after challenge with the wild-type virus. These results indicate that S-PRV-013 is an effective vaccine strain which permits 10 vaccines to be distinguished from animals infected with wild-type virus by a sample serum diagnostic assay.

#### EXAMPLE 7

##### S-PRV-014

15 S-PRV-014 is a pseudorabies virus that has a deletion in the gpX coding region. The gene for *E. coli* beta-galactosidase was inserted in place of the gpX gene and is under the control of the endogenous gpX promoter.

The following procedures were used to create S-PRV-014 by homologous recombination. The flanking PRV homology regions were from the cloned BamHI #10 fragment which contains the gpX promoter, and from the cloned BamHI 20 #7 fragment extending from the NdeI site to the BamHI site (Figure 9). The NdeI site was filled in according to the POLYMERASE FILL-IN REACTION, and the beta-galactosidase gene was inserted between the BamHI #10 and BamHI #7 fragments. This construct positioned the beta-galactosidase gene behind the gpX promoter and the gpX poly A signal sequence with a deletion of almost all of the coding region of gpX. The plasmid DNA and DNA from wild-type PRV were mixed and transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS 25 procedure. The recombinant virus was screened and purified from the transfection stock by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure.

The resulting virus from this screen was designated S-PRV-014 and has been deposited with the ATCC under Accession No. VR 2135. It contains the beta-galactosidase gene in place of the gpX coding region as determined by PREPARATION OF HERPESVIRUS DNA followed by SOUTHERN BLOTTING DNA. The expression of the beta-galactosidase gene was confirmed by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS test, and by the o-nitrophenylgalactopyranoside substrate assay (33). The structure of this virus is shown in Figure 9D.

#### EXAMPLE 8

##### S-PRV-016

35 S-PRV-016 is a pseudorabies virus that has a deletion in both repeat sequences, and a deletion in the gpX coding region. The gene for *E. coli* beta-galactosidase was inserted in place of the gpX gene and is under the control of the endogenous gpX gene promoter.

40 The following procedures were used to construct S-PRV-016 by homologous recombination. The flanking PRV homology regions were from the cloned BamHI #10 fragment which contains the gpX promoter, and from the cloned BamHI #7 fragment extending from the NdeI site to the BamHI site (Figure 9). The NdeI site was filled in according to the POLYMERASE FILL-IN REACTION, and the beta-galactosidase gene was inserted between the BamHI #10 and BamHI #7 fragments. The construct positioned the beta-galactosidase gene behind the gpX promoter and the gpX poly 45 A signal sequence with a deletion of almost all of the coding region of gpX. The plasmid DNA and DNA from S-PRV-001 were mixed and transfected according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. The recombinant virus was screened and purified from the transfection stock by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure.

The resulting virus from this screen was designated S-PRV-016 and has been deposited with the ATCC Accession 50 No. VR 2136. It contains the beta-galactosidase gene in place of the gpX coding region as determined by PREPARATION OF HERPESVIRUS DNA followed by SOUTHERN BLOTTING OF DNA. The expression of the beta-galactosidase gene was confirmed by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS test, and by the o-nitrophenylgalactopyranoside substrate assay (33). The structure of this virus is shown in Figure 9E.

#### EXAMPLE 9

##### S-PRV-020

S-PRV-020 is a pseudorabies virus that contains a deletion in the TK gene, a deletion in the repeat regions, and a

deletion of the gpX gene, with an insertion of the swine parvovirus B capsid protein gene into the gpX region.

For cloning the swine parvovirus B gene, the NADL-8 strain double-stranded replicative-form DNA was purified from swine parvovirus infected cells and was supplied by Dr. T. Molitor, University of Minnesota. The parvovirus NADL-8 DNA was cloned into the *E. coli* plasmid pSP64 by methods detailed in (15). The DNA was partially sequenced to allow the determination of the start and the end of the major capsid protein gene, the B gene. Identification was confirmed by comparison of related sequences in the rat H1 parvovirus capsid gene (28,29). The sequence of the swine parvovirus B gene is shown in Fig. 11A and 11B.

The  $P_{gpX}$  glycoprotein X (gpX) gene promoter was used to express the B gene and the gpX poly A signal sequence was used to terminate transcription. The parvovirus B gene from the AccI site at nucleotide #391 to the RsaI site at nucleotide #2051 was cloned between the BamHI and NdeI site of gpX (see Figure 7A and B). Plasmid pSY864 contained this fragment of the parvovirus B gene flanked by the gpX signal sequence as shown in Figure 13. It was used as the homologous DNA to promote homologous recombination between S-PRV-013 DNA and the plasmid DNA to facilitate the incorporation of the B gene into the PRV genome. The plasmid DNA and S-PRV-013 DNA were mixed and transfected together according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. The recombinant virus was screened and purified for the transfection stock by the BLUGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure with the following modification. Because the parental S-PRV-013 contained the beta galactosidase gene, it generated blue plaques in the screening procedure. Since the parvovirus B gene would replace the beta galactosidase gene in the virus, the plaques with this insert would appear colorless. Therefore, colorless plaques were picked and analyzed during this screening. A virus that contained the B gene was isolated from this screening and was designated S-PRV-020. S-PRV-020 has been deposited with the ATCC under Accession No. VR 2137.

DNA from S-PRV-020 was isolated by the PREPARATION OF HERPESVIRUS DNA procedure and used to confirm the insertion of the parvovirus B gene according to the SOUTHERN BLOTTING OF DNA procedure using the B gene as a probe. The test showed that the parvovirus B gene has been incorporated into the PRV genome as expected. The structure of S-PRV-020 is shown in Figure 13C.

#### EXAMPLE 10

##### S-PRV-025

The cloning of the B gene and construction of these signal sequences onto the B gene are described in Example 9 and are shown in Figure 12.

The DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS was used to insert the parvovirus B gene into PRV. The plasmid pSY957 containing the B gene was mixed with S-PRV-002 DNA and they were cut with restriction enzyme XbaI. The DNA mixture was ligated as described in the method and the DNA was transfected into Vero cells. A virus that contained the B gene was isolated from the transfection stock of virus and was designated S-PRV-025. S-PRV-025 has been deposited with the ATCC under Accession No. VR 2138.

DNA from S-PRV-025 was isolated by the PREPARATION OF HERPESVIRUS DNA procedure and used to confirm the insertion of the parvovirus B gene according to the SOUTHERN BLOTTING OF DNA procedure using the B gene as a probe. The test showed that the parvovirus B gene has been incorporated into the PRV genome as expected. The structure of S-PRV-025 is shown in Figure 14.

#### EXAMPLE 11

##### S-PRV-029

S-PRV-029 is a pseudorabies virus that has a deletion in the junction region between the unique long region and the internal repeat of PRV, and a deletion in the gpX gene in the unique short region. The *E. coli* beta-galactosidase gene under the control of the gpX promoter and polyadenylation signals has been inserted into both deletions in S-PRV-029.

To construct this virus, the Sall #1 fragment of PRV was first cloned. PRV DNA was prepared and then cut with Sall restriction enzyme. The cut DNA was electrophoresed on an agarose gel and the largest Sall band (15 kb) was purified from the gel (see PHENOL EXTRACTION OF DNA FROM AGAROSE). The purified DNA was ligated into the plasmid pSP64 (see LIGATION) and the DNA mixture was used to transform *E. coli* HB101 according to Maniatis et al. (1). The Sall #1 clone was mapped for restriction sites.

The homologous recombination procedure was used to create S-PRV-029. The exact position of the junction region was determined by sequencing the DNA from the Sall #1 fragment. It was found that the junction region was positioned between two StuI sites (see Figure 15B). Two fragments of DNA from the Sall clone were used to create the homology vector for recombination. One was a fragment from BamHI #9' from StuI to BamHI and the other was from BamHI to

5 StuI (Figure 15B).

The *E. coli* beta-galactosidase gene was previously engineered to contain the gpX promoter And polyadenylation signals as described for S-PRV-013. To put this B-galactosidase gene into the junction region clone, a HindIII linker was first inserted into the StuI site between the BamHI #8 and BamHI #8', and into this HindIII site was cloned a HindIII fragment containing the beta-galactosidase gene with the gpX signals.

10 The resulting plasmid plus wild-type PRV DNA were transfected into Vero cells by the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. A virus was isolated from the transfection stock that contained the beta-galactosidase gene inserted into both the junction deletion (Fig. 15B) and the gpX deletion (Fig. 15A) due to the presence of homology to both of these regions in the plasmid. This virus was purified by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure and was designated S-PRV-029. S-PRV-029 has been deposited with the ATCC under Accession No. VR 2139.

15 S-PRV-029 was shown to be expressing beta-galactosidase by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS procedure and the o-nitrophenylgalactopyranoside assay (33). The structure of this virus is shown in Figure 15C.

EXAMPLE 12

S-IBR-002

20 S-IBR-002 is an IBR virus that has a deletion of approximately 800 bp in the repeat region of the genome. This deletion removes the only two EcoRV restriction sites on the virus genome and an adjacent BglII site (Figure 16).

To construct this virus, the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUSES was performed. Purified IBR DNA (Cooper strain) digested with EcoRV restriction enzyme was mixed with Drai-restriction enzyme-digested plasmid DNA containing the beta-galactosidase gene under the control of the HSV-1 25 TK promoter. After ligation the mixture was used to transfet animal cells and the transfection stock was screened for recombinant IBR virus by the HYBRIDIZATION SCREEN FOR RECOMBINANT HERPESVIRUSES procedure. The final result of the purification was the recombinant IBR designated S-IBR-002. It was shown by Southern hybridization that this virus does not carry any foreign genes. Restriction enzyme analysis also showed that the insertion sites (EcoRV) at both repeats were deleted. Figure 15 shows the restriction map of the EcoRI B fragment which contains the 30 EcoRV restriction sites and the map of S-IBR-002 which lacks the EcoRV sites. S-IBR-002 has been deposited with the ATCC under Accession No. VR 2140.

EXAMPLE 13

S-IBR-004

S-IBR-004 is an IBR recombinant virus carrying an inserted foreign gene, Tn5 NEO (aminoglycoside 3'-phosphotransferase) gene, under the control of the pseudorabies virus (PRV) glycoprotein X promoter.

To construct this virus, the HindIII K DNA fragment from wild type IBR virus was cloned into the plasmid pSP64 at the HindIII site. This plasmid was designated pSY524. A map of the HindIII K fragment is shown in Figure 17. The DNA from the Xhol site to the HindIII site and containing the NdeI site from pSY524 was cloned into plasmid pSP65 and called pSY846. The NdeI to EcoRI fragment was removed from pSY846 by digestion with NdeI and EcoRI restriction enzymes, followed by POLYMERASE FILL-IN REACTION and LIGATION. The resulting plasmid was called pSY862. The plasmid pNEO (P.L. Biochemicals, Inc.) contains the aminoglycoside 3'-phosphotransferase (NEO) gene and confers resistance to ampicillin and neomycin on *E. coli* hosts. The coding region of this gene (BglII-BamHI fragment) was isolated and cloned between the PRV gpX promoter and the HSV-Tk poly A sequence in a plasmid called pSY845.

The NEO gene construct in pSY845 was excised with HindIII, made blunt ended by the POLYMERASE FILL-IN REACTION, and cloned into the SacI site of plasmid pSY862. The final product was called pSY868.

Wild type IBR DNA was mixed with pSY868 DNA and the mixture was transfected into rabbit skin cells to generate recombinant IBR. The recombinant IBR virus carrying a functional NEO gene was then isolated and purified according to the SELECTION Of G418 RESISTANT VIRUS method.

S-IBR-004 recombinant IBR was shown to express the NEO gene by the fact that cells infected with this virus were resistant to the toxicity of G418. A detailed map of the plasmid construction is shown in Figure 17. The structure of S-IBR-004 is also shown in Figure 17. S-IBR-004 has been deposited with the ATCC under Accession No. VR 2134.

EXAMPLE 14S-IBR-008

5 S-IBR-008 is an IBR virus that has a deletion in the short unique region, and an insertion of the bovine rotavirus glycoprotein 38 (gp38) gene in the XbaI site in the long unique region.

First the bovine rotavirus gp38 gene was engineered to contain herpesvirus regulatory signals as shown in Figure 18. This was accomplished by cloning the gp38 gene BamHI fragment contained in pSY1053 between the BamHI and BglII sites in pSY1052. The resulting plasmid, pSY1023, contained the PRV gpX promoter in front of the gp38 gene, and the HSV-1 TK polyadenylation signal behind the gp38 gene. The entire construct was flanked by XbaI sites to allow for the insertion of the XbaI fragment into IBR by direct ligation.

10 S-IBR-004 was the starting virus for the generation of S-IBR-008. S-IBR-004 DNA and pSY1023 DNA were mixed together, cut with XbaI, and transfected into rabbit skin cells according to the DIRECT LIGATION FOR GENERATING RECOMBINANT HERPESVIRUS procedure. The transfection stock was screened for recombinant virus by the ANTI-15 BODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure using antibodies prepared against the rotavirus gp38 protein.

15 One of the viruses purified by this screen was S-IBR-008, which has the following characteristics. It contains the rotavirus gp38 gene plus the plasmid DNA inserted into the XbaI site in the long unique region of the virus genome, but no longer contains the NEO gene of parent S-IBR-004 in the unique short region. In fact, a small deletion was created 20 in the unique short region at the location of the NEO gene, as evidenced by the absence of an XbaI site at this location in S-IBR-008.

25 S-IBR-008 was shown to be expressing the rotavirus gp38 gene by analysis of RNA transcription in infected cells, and by the ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure using antibodies specific for the gp38 gene. S-IBR-008 has been deposited with the ATCC under Accession No. VR 2141, and its structure is shown in Figure 18.

EXAMPLE 15Herpes Virus of Turkeys

30 Herpes virus of turkeys (HVT) is another herpesvirus that is similar in organization and structure to the other animal herpesvirus examples described above. The restriction enzyme map of HVT has been published (34). This information was used as a starting point to engineer the insertion of foreign genes into HVT. The BamHI restriction map of HVT is shown in Figure 19A. From this data, several different regions of HVT DNA into which insertions of foreign genes could 35 be made were targeted. The foreign gene chosen for insertion was the *E. coli* beta-galactosidase gene (beta-gal), which we have used in PRV. The promoter was the PRV gpX promoter. The beta-gal gene was inserted into the unique long region of HVT, specifically into the XbaI site in the BamHI #16 (3300bp) fragment, and has been shown to be expressed in an HVT recombinant by the formation of blue plaques using the substrate Bluogal. Similarly, the beta-gal gene has been inserted into the SalI site in the repeat region contained within the BamHI #19 (900bp) fragment.

40 These experiments show that HVT is amenable to the procedures described within this application for the insertion and expression of foreign genes in herpesviruses. In particular, two sites for insertion of foreign DNA have been identified (Figs. 19B and 19C).

EXAMPLE 16Methods for Constructing An Attenuated Herpesvirus Containing A Foreign DNA Insert

45 Applicants contemplate that the procedures disclosed herein which have been utilized to attenuate and insert foreign DNA sequences into PRV, IBR and HVT may be suitable for constructing other herpesviruses which are attenuated or contain inserted foreign DNA sequences which are translated into amino acid sequences in a host or both. Equine herpesvirus-1 (EHV), canine herpesvirus-1 (CHV), feline herpesvirus-1 (FHV) or any animal herpesvirus whose genomic structure is related to these viruses are contemplated to be amenable to these methods. More specifically, the following procedures may be followed to construct such viruses.

50 GROW ANIMAL HERPESVIRUS IN CELL CULTURE. Established cell lines or primary cells may be used. The methodology for the growth of these viruses exists in the literature and does not require new art. EHV grows in Vero cells, CHV grows in Madin Darby canine kidney cells and FHV grows in Crandell feline kidney cells.

55 PURIFY HERPESVIRUS DNA. The procedure disclosed herein for purifying herpesvirus DNA was successful for all herpesviruses tested, including PRV, IBR, HVT and cytomegalovirus, and is a general method applicable to all herpesviruses.

CLONE RESTRICTION FRAGMENTS. The cloning of herpesvirus restriction fragments is a state of the art recombinant DNA procedure and is described in Maniatis et al. (1).

MAP RESTRICTION FRAGMENTS TO GENOME. It is useful to have a restriction enzyme map of the virus genome to identify and select regions for deletion and insertion. Such maps are available for PRV and IBR, and partially for HVT. A map exists for EHV, but not for CHV or FHV. The creation of this map does not require any new technology and is detailed in Maniatis et al. (1).

IDENTIFY RESTRICTION FRAGMENTS THAT CORRESPOND TO THE REPEAT REGION. The identification of repeat regions requires the SOUTHERN BLOTTING PROCEDURE as detailed in the methods section. Clones of the repeat region hybridize to multiple bands in a restriction enzyme digest due to the fact that they are repeated in the virus genome. This feature, coupled with their location in the genome, are diagnostic of repeat regions.

MAKE DELETION IN REPEAT REGION CLONE. Genetic information in the repeat region is duplicated in the other copy of the repeat in the genome. Therefore one copy of the repeat region is nonessential for replication of the virus. Hence the repeat region is suitable for deletions and insertions of foreign DNA. After the repeat region is cloned and mapped by restriction enzymes, enzymes may be chosen to engineer the repeat deletion and to insert foreign DNA. It is obvious to one skilled in the art that enzyme sites will exist in a given stretch of DNA and that they can be found by analysis. The methodology involves RESTRICTION DIGESTION OF DNA, AGAROSE GEL ELECTROPHORESIS OF DNA, LIGATION and cloning in bacterial cells as detailed in the methods section and in Maniatis et al. (1).

MAKE INSERTION OF MARKER GENE INTO DELETION IN REPEAT REGION CLONE. The methodology of this insertion is that described in Maniatis et al. (1) for the cloning of genes into bacteria. What is not obvious prior to the present disclosure is which marker genes to use that will be active in a herpesvirus, nor which signal sequences to use for the expression of foreign genes in these herpesviruses. The *E. Coli* beta-galactosidase gene and neomycin resistance gene under the control of the HSV-1 ICP4 promoter, the PRV gpX promoter or the HSV-1 TK promoter have been used. The gpX promoter, in particular, works in PRV, IBR, and HVT. The other promoters have also worked in more limited testing.

TRANSFECTION WITH MARKER GENE CLONE + HERPESVIRUS DNA. The intent of this procedure is to put into the same cell the intact herpesvirus DNA and the repeat region clone with the deletion and containing the marker gene. Once these two DNAs are present in the same cell, normal mechanisms of homologous recombination ensure that a recombination will occur between the homologous regions in the clone and the same region in the herpesvirus DNA, thus substituting the marker gene for the deleted regions in the virus, with frequency of about 1%. The technique involves the TRANSFECTION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUSES as detailed in the methods section.

PURIFY HERPESVIRUS DNA. Herpesvirus DNA may be purified according to the methods described above.

SELECT RECOMBINANT PLAQUE. All the herpesviruses contemplated by this invention form plaques (foci of infection in cell culture) that enable their purification. A plaque results from infection by a single virus particle. Thus picking a single plaque selects for the progeny of a single recombinational event. This technical feat requires a method to identify which plaque to pick. The methods used herein include SOUTHERN BLOTTING OF DNA to pick the plaque based upon the presence of the inserted gene, ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS to pick the plaque based upon the presence of protein made from the gene, BLUGAL SCREEN FOR RECOMBINANT HERPESVIRUSES to pick a plaque that expresses the marker gene beta-galactosidase or G-418 SELECTION TO PURIFY RECOMBINANT HERPESVIRUSES to pick the plaque by its ability to form in the presence of the antibiotic G-418. The first two methods are applicable to any gene; the latter two are specific for the beta-galactosidase gene and neomycin resistance gene respectively. The biology of these screening and selection systems is such that they are applicable to any herpesvirus, including EHV, CHV, FHV, and any animal herpesvirus related to them.

PURIFY RECOMBINANT VIRUS. This procedure involves multiple plaque purifications in succession to completely purify the recombinant virus away from the parental virus. The screening is applied at each step to choose the plaque with which to continue. The procedures are known to those skilled in the art of virology.

Multivalent vaccines for animals may be constructed by inserting a foreign antigen gene into a herpesvirus. The procedures and methodology are very analogous to those used for the initial insertion of the marker gene into the virus and may be performed as follows.

SUBSTITUTE FOREIGN ANTIGEN GENE FOR MARKER GENE IN REPEAT CLONE. This is a cloning experiment that involves putting the antigen gene behind the same herpesvirus promoter used with the marker gene and inserting this construction into the same identical deletion in the repeat clone. The methods for this cloning are described in Maniatis et al. (1).

TRANSFECTION WITH ANTIGEN CLONE + RECOMBINANT HERPES DNA CONTAINING MARKER. The marker gene that is already present in the herpesvirus genome may be used to aid in the selection of the new recombinant. For example, it has proven useful to select white plaques instead of blue ones to test for the absence of beta-galactosidase in this step. One reason for the present of a white plaque is the replacement of the beta-galactosidase gene with the foreign antigen gene by homologous recombination (the desired outcome). Continued screening for this new recombinant by the SOUTHERN BLOTTING PROCEDURE or by the ANTIBODY SCREEN FOR RECOMBINANT HER-

PESVIRUS becomes more focused, less time-consuming and specifically identifies the recombinant of interest.

ISOLATE RECOMBINANT HERPES DNA. The transfection procedure requires intact infectious herpesvirus DNA. Recombinant herpesviruses which include an inserted marker gene may be used. The isolation of herpesvirus DNA procedure is equally applicable to these recombinant viruses.

SCREEN TRANSFECTION STOCK FOR FOREIGN GENE INSERTION. Screening methods have been described above. They are a combination of indirect methods (screening for the absence of the marker) as well as direct methods (SOUTHERN BLOT for the antigen gene, and ANTIBODY SCREEN for the expressed protein). The methods may be applied sequentially during the purification of the virus.

PURIFY RECOMBINANT VIRUSES CONTAINING FOREIGN ANTIGEN GENE. The recombinant virus containing the foreign antigen gene may be purified according to the procedures described above.

This sequence of steps, along with the methods and examples described herein, enable anyone skilled in the art to successfully practice this invention with any animal herpesvirus.

#### EXAMPLE 17

The present invention involves the use of genetically engineered herpesviruses to protect animals against disease. It was not apparent at the outset of research which deletions in herpesviruses would serve to attenuate the viruses to the proper degree so as to render them useful as vaccines. Even testing vaccine candidates in animal models, e.g. mouse, does not serve as a valid indicator of the safety and efficacy of the vaccine in the target animal species, e.g. swine. To illustrate this point more clearly, Table VII shows summary data of the safety and efficacy of various pseudorabies viruses which were constructed and tested in swine according to the VACCINATION STUDIES IN SWINE procedure.

TABLE VII

SUMMARY OF STUDIES CONDUCTED IN PIGS WITH VARIOUS PSEUDORABIES VIRUS CONSTRUCTS					
Construct (Deletions/Insertions) <sup>1</sup>	Number of Pigs	Age of Pigs	Post-Vaccination		Percent Protection Against Challenge
			Antibody Range	Clinical Signs	
S-PRV-001 (A)	9	4-6 weeks	1:32->1:64	Yes (22%)	Not Done
S-PRV-002 (A,B)	12	4-6 weeks	1:4-1:64	None	100
S-PRV-003 (B)	8	4-6 weeks	<1:2-1:16	None	50
S-PRV-004 (B,C)	6	4-6 weeks	1:4-1:32	None	64
S-PRV-010 (A,B,E)	30	4-6 weeks	<1:2-1:16	Yes (13%)	100
	30	3-4 days	1:4-1:64	Yes (13%)	100
S-PRV-013 (A,B,D,E)	23	4-6 weeks	<1:2-1:8	None	100
	25	3-4 days	1:4-1:64	None	100
S-PRV-014 (D,E)	5	4-6 weeks	1:4-1:8	Yes (40%)	100
S-PRV-016 (A,D,E)	5	4-6 weeks	1:4-1:8	None	100

<sup>1</sup>A-Repeats; B-TK; C-Junction; D-gpX; E-beta-galactosidase insert

The eight constructs that have been tested have the following deletions and insertions in the genome of the virulent Shope strain of PRV: S-PRV-001 has a deletion in both repeat regions; S-PRV-002 has a deletion in both repeat regions and in the thymidine kinase gene; S-PRV-003 has a deletion in the thymidine kinase gene; S-PRV-004, S-PRV-010, S-PRV-013, S-PRV-014 and S-PRV-016 are described in Example #'s 1, 3, 6, 7 and 8 respectively.

A superior vaccine product must not produce clinical signs in 3-4 day old piglets (the more sensitive age), and give 100% protection in pigs of all ages. From Table VII, it is apparent that each vaccine candidate provided some degree of attenuation and protection in swine, but each vaccine provided a unique response. The best vaccine candidate from this list to date is S-PRV-013, which contains three deletions; one in the repeat region; one in the TK gene, and one in the gpX gene. The utility of this combination of deletions was unexpected. These results are novel, unpredicted, and useful in the selections of a superior pseudorabies vaccine product.

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Claims

1. A recombinant herpesvirus of turkeys comprising a foreign DNA inserted into a non-essential region of the herpesvirus of turkeys viral genome, which is capable of being expressed in a host cell infected with the herpesvirus of turkeys.
- 5 2. The recombinant herpesvirus of turkeys of claim 1, wherein the foreign DNA is inserted into the BamHI 16 region of the herpesvirus of turkeys viral genome.
- 10 3. The recombinant herpesvirus of turkeys of claim 2, wherein the foreign DNA is inserted within the Xhol site of the BamHI 16 region of the herpesvirus of turkeys viral genome.
- 15 4. The recombinant herpesvirus of turkeys of claim 1, wherein the foreign DNA sequence encodes a polypeptide.
5. The recombinant herpesvirus of turkeys of claim 4, wherein the polypeptide is antigenic.
- 15 6. The recombinant herpesvirus of turkeys of claim 4, wherein the polypeptide is *E. coli* beta-galactosidase.
- 20 7. A vaccine which comprises an effective immunizing amount of the recombinant herpesvirus of turkeys of claim 1 and a suitable carrier.

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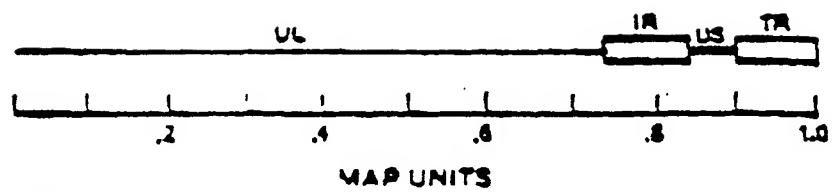
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Figure 1

A.



B.

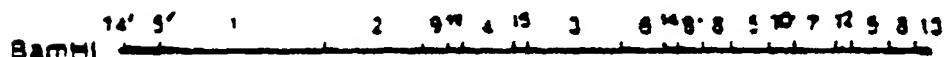
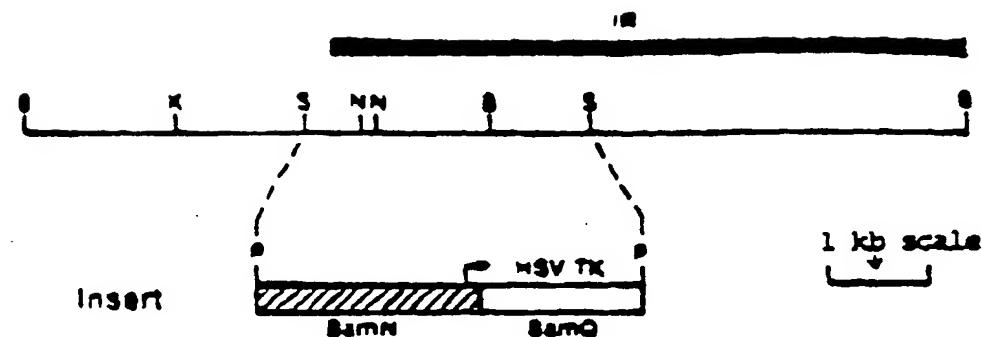


Figure 2



B.



C.



Figure 3

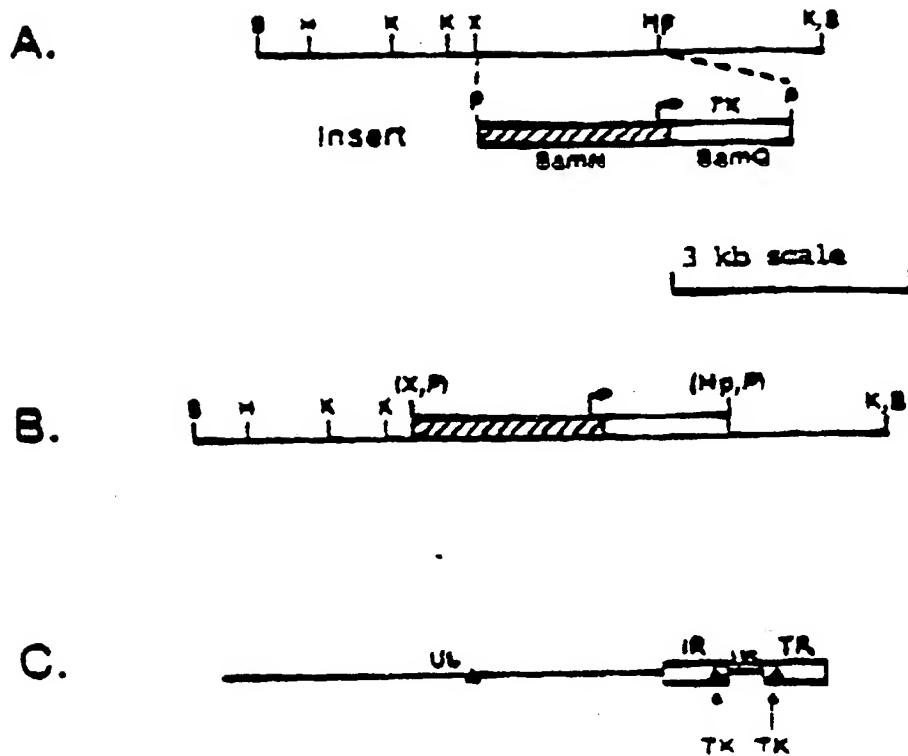


Figure 4

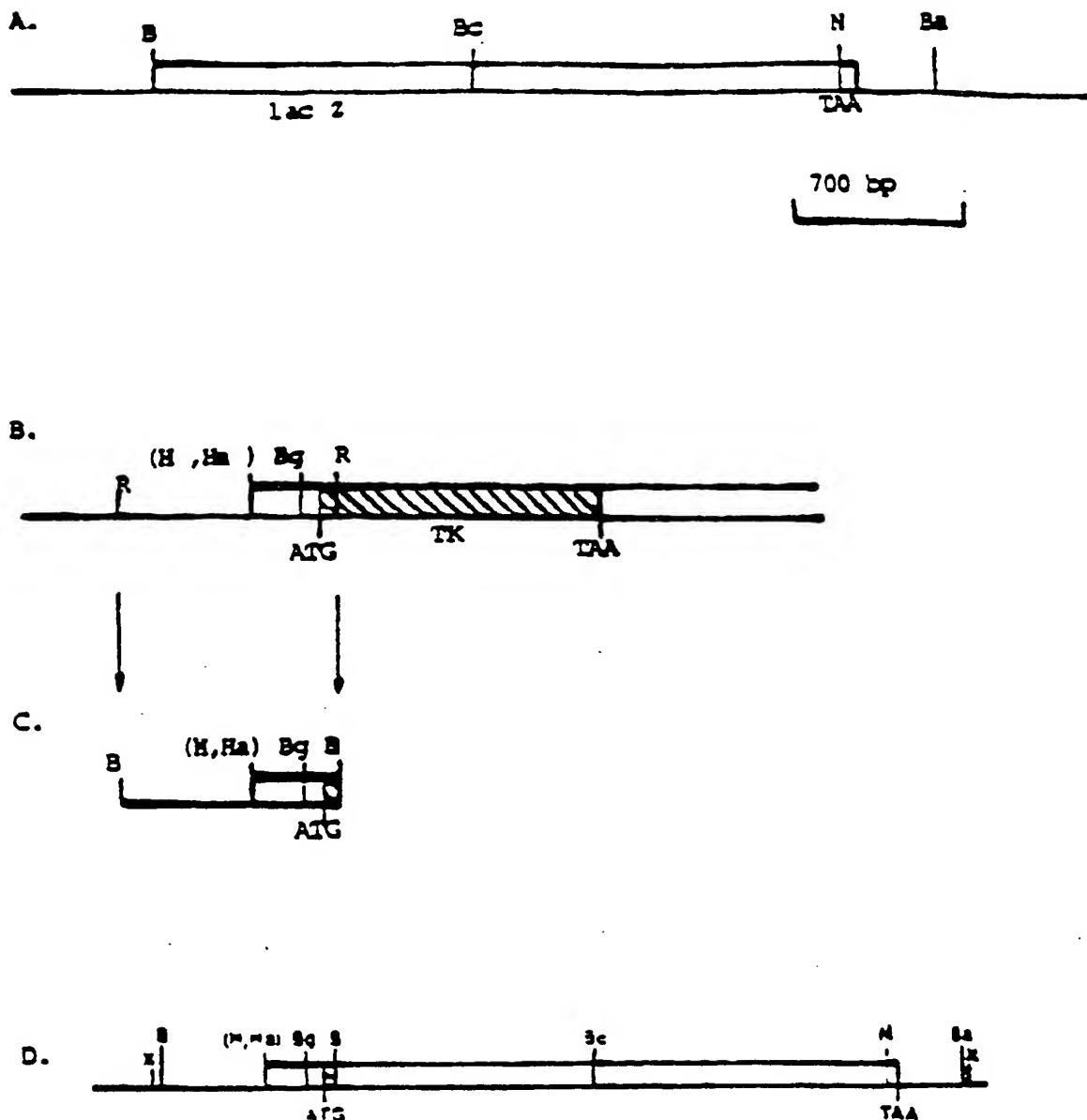


Figure 5

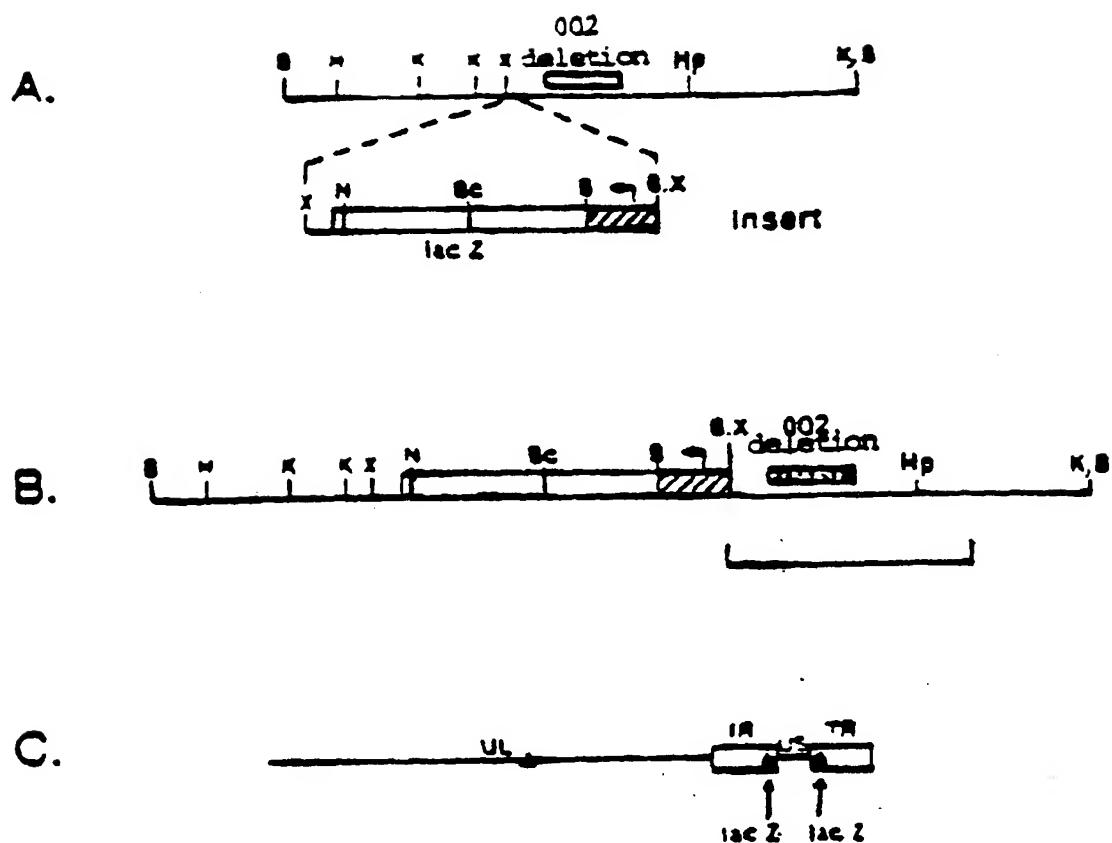


Figure 6

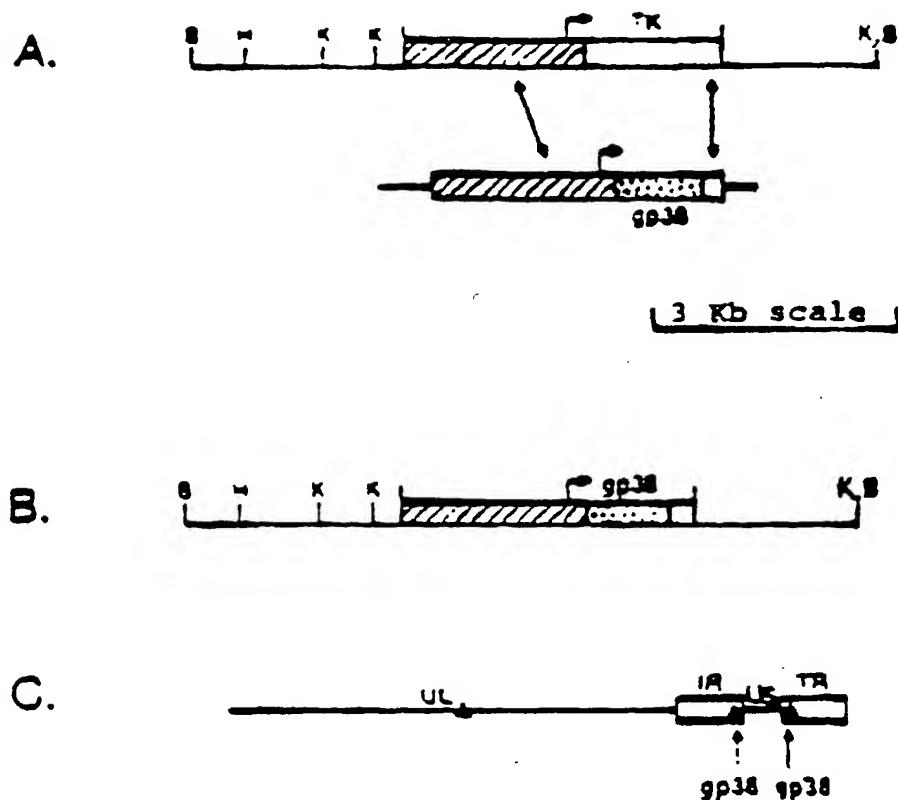


Figure 7

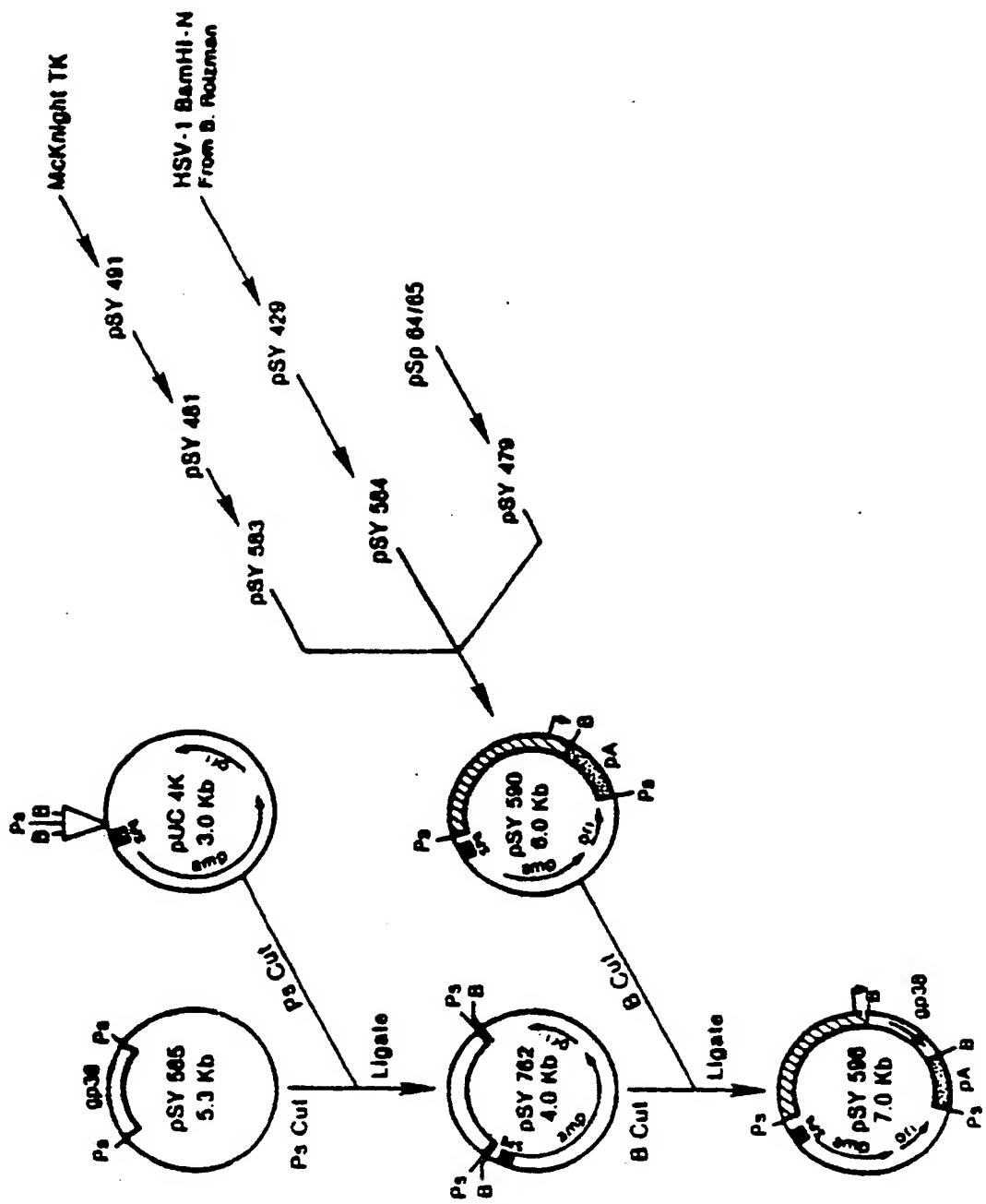


Figure 8

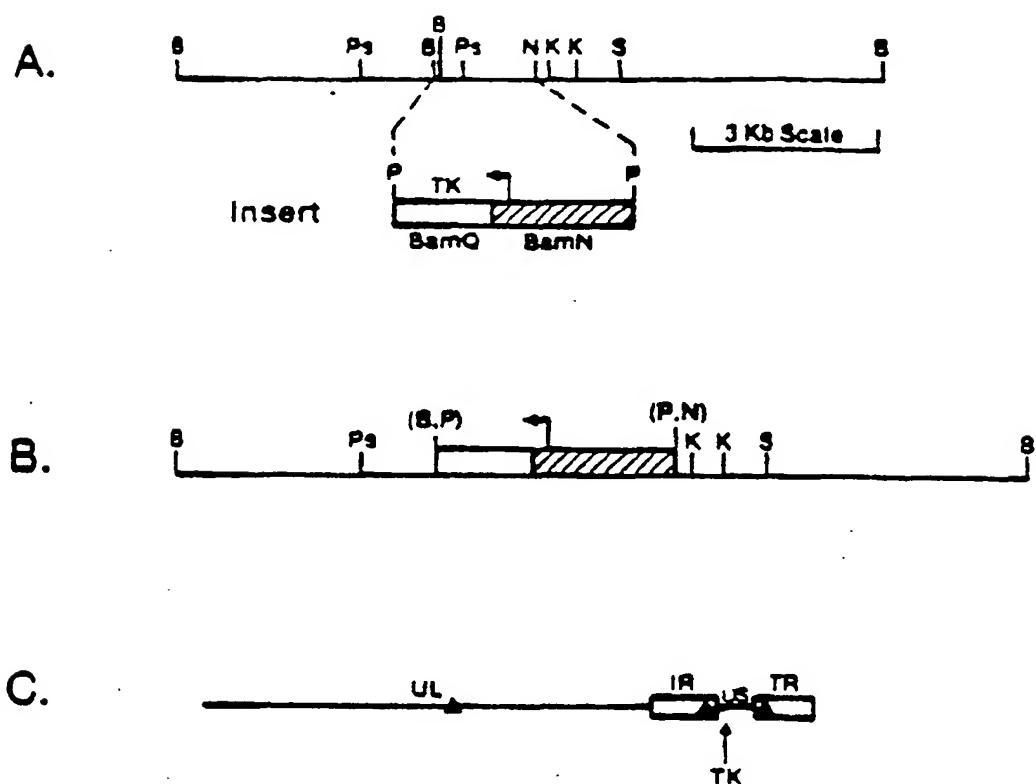


Figure 9

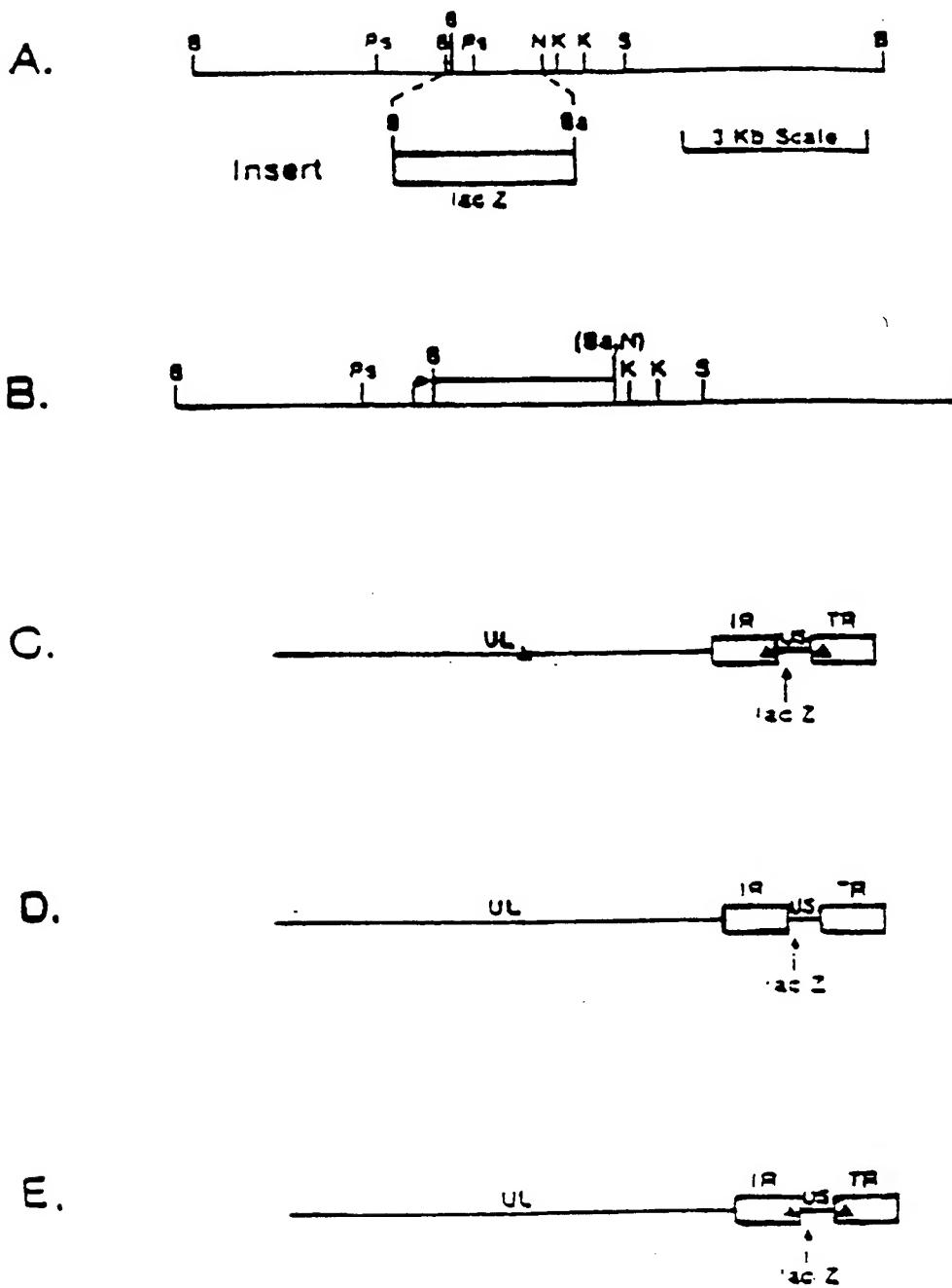


Figure 10A

Figure 10B

Figure 11A

1 CTCATGAA ATTCAATAAA GAACTGAAAC ACGCAAAAAGA CTACGGAGGT AAAATTGGAC  
 61 ATTACTCTT CAGAGCAAG CGTCCTTTG CTCCAAAACT CTAGAAACA GACTCACCA  
 121 CTACATCTA AGAACACAGC GTAGAGAT CGCCGAGAA AGACCCAGGG TCTAAACCA  
 181 CAGGAAAG ACCTGCTCA AGACATTTT TTATMAACTT AGCTAAAAA AAAGCTAAAG  
 241 GGACATCTA TACAAACTT AACCTCAATGA GTCAAAATGT GGAAACACAC AACCCATTAA  
 METS-εrgluuuvv4-1GluGln18 AsnPro0...  
 301 ATCCAGGCCAC TGAATCTCT CCACAGCAA ATGAATCTGG GGGGGGGG GGGGGGGG  
 AccI  
 361 GGGCTAGGGG TCCTGGGGG CTTCGGCTGT CTACAGGTAG TTTCAATAT CAAACAGAA  
 421 TTCAAATACTT GGGGGGGG TTGGCTTAGAA TCACTGGCACA CGCATCAGA CTCAATACATC  
 RsaI  
 481 TAAATATGCC AGAACACGAA ACATAACAAAC GATAACATGT ACTAAATTCG GAAATCAGGGG  
 RsaI  
 541 TGGCCGACA ATGGCTACAA GACGATGCAAC ACACACAAAT GGTAAACACTT TGGTCACAA  
 601 TAGATCGTAA CGCATGGGA GTCCTGGTCA ATCCAGGGAA CTGGCAATTAA TATCCAAAC  
 RsaI  
 661 ACATGACAGA ATTAACCTTA GTTAGTTTG AACAAAGAT ATTCAATGTA GTACTTAAAA  
 721 CAAATACAGA ATCAGCAACC TCAACCAT CCAAAATATA TAATAATGAT CTAACTGGAA  
 781 GCTTAATGGT CGCACTAGAC ACCATAACA CACTTCCATA CACCCAGGA GCACCTAGAA  
 841 GTCGAACACTT TGGTTTTAT CCATGGTAC CTACAAACCC AACTCATAC AGATATTAC  
 901 TATCATGCAAT CAGAAACCTA ATCCACCAA CATAACCTGG ACATACAGA CAAATCACAA  
 RsaI

Figure 11b

961 ACTCAATACA AACAGGACTA CACAGTGACA TTATGTTCTA CACATAGAA AATGCACTAC  
 1021 CAACTCATCT TCTTAAAGACT GGAGATGAAAT TCTCCACAGG ATATATACAC TTGACACAA  
 1081 ACCATTAA ATTAACTCAC TCAATGCCAA CAAACAGATC TCTAGGACTG CCTCCAAAC  
 1141 TACTAATGA ACCTACCAAA GAGGGAGACC AACACCCAGG AACATCAAA GCAACTAAAC  
 1201 CAGAAAGG TTATCACCA ACAAATATAA ATAGCTAAC CAGAGCAAA GCACTTGGCC  
 1261 CAGCTCAGT AGGATATATACACCACTACAA TGAAATTGCA CTACTCCAACT GGTGGACCAT  
 1321 TTCTAACTCC TATAGTACCA ACAGCAGACA CACATATTAA TGATGAA CCAATATGGTC  
 1381 CTATAGATT TACAAATGTT TACCAACATG GACACTAAC CACATCTCA CAGAGCTAG  
 1441 AACAGTACAC ATTCAATCCAA CAAAGTAAAT GTCGAGAGC TCCAAAGCAA CAATTAAATC  
 1501 AACAGGCCAC ACTAAACCTAA GAAATACAA ATAAATGGAC ACCTTTACCT TCAAGATCCAA  
 1561 TAGGAGGAA ATCTAACAG CATTTCATGAA ATACACAA TACATATGGA CCATTAAACAG  
 1621 CACTAAACAA TACTGCACTT GTATTCCAA ATGGTCAAT ATGGGATAAA GAACTTGATA  
 1681 CAGCTCTAA ACCTAGACTA CATTCTTACAG CTCCATTGTGT TTCTAAACAC ATCCACCCAG  
 1741 GACAACTATT TCTTAAATTA GCAACCAACCC TAAAGATGAA TTACAGATGA GACTCTCC  
 1801 AACAACTAG AATTAATCTT GATTCAAATCTT TTGGTGGM AGGAACACTA ACGTTCAAG  
 1861 CAAATATGAG ATCCAGTAACT ATGGGAACCC CTATTCAACA ACACACAAAC ACGGAGAA  
 1921 AACATTGAA ATATATTCTT ACATATTCG CTGCTATCAA ATGTTCCAA GAAATATCAC  
 1981 AACCTATACC AACGAAATTA TACTGAAAT AACCTCTAA ATAAAATCTC AGTTACTGG  
 .. LeuileP<sup>r</sup>-oArglyleu Tyr---  
 2041 TTATCTGTT ACTACTATCA TC

Rsa I  
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Figure 12

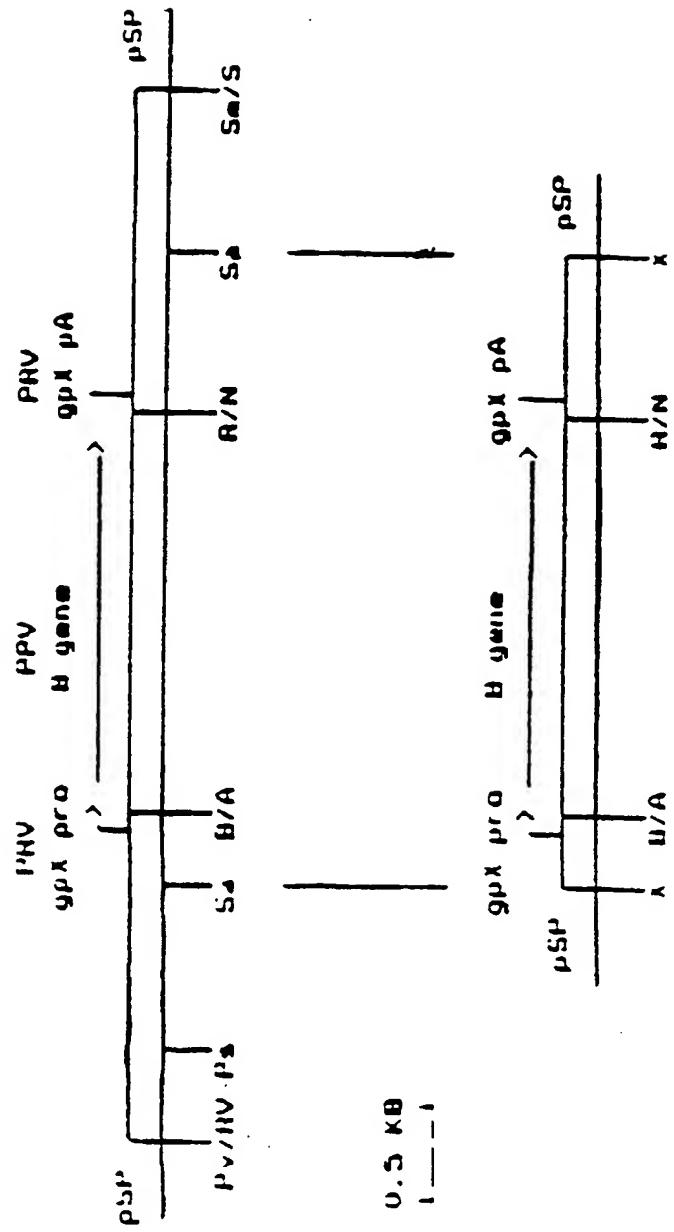


Figure 13

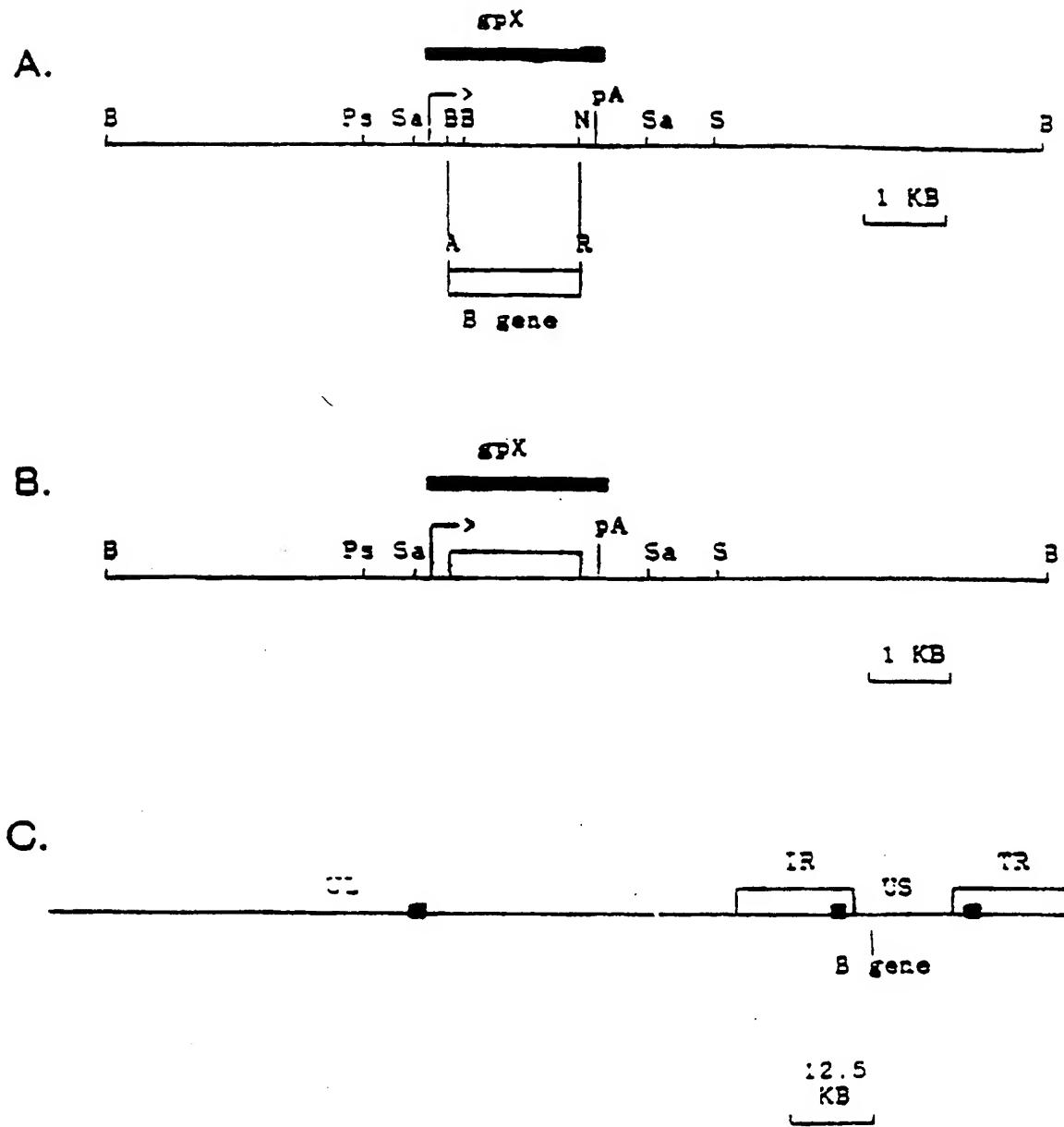


Figure 14

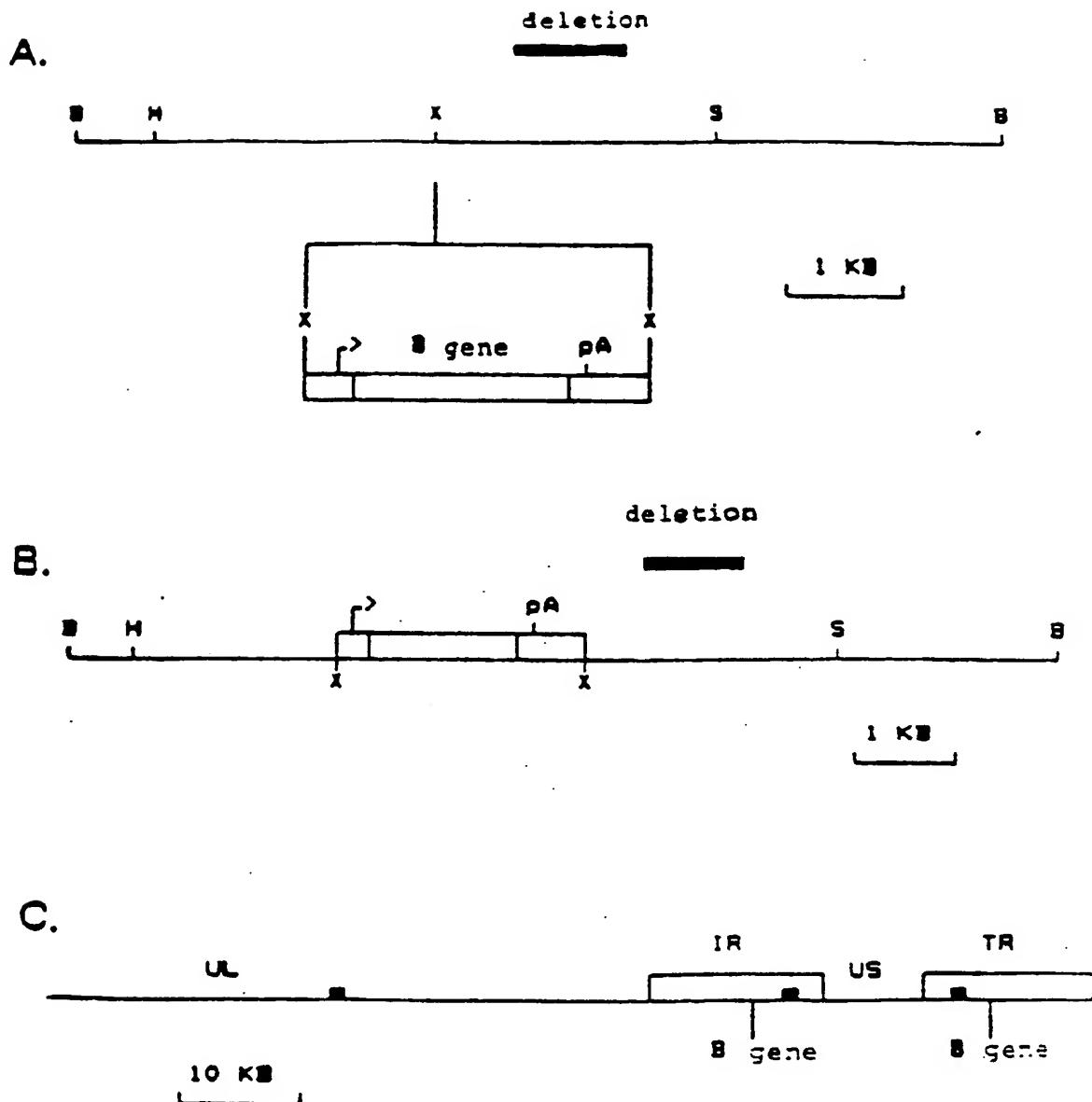


Figure 15

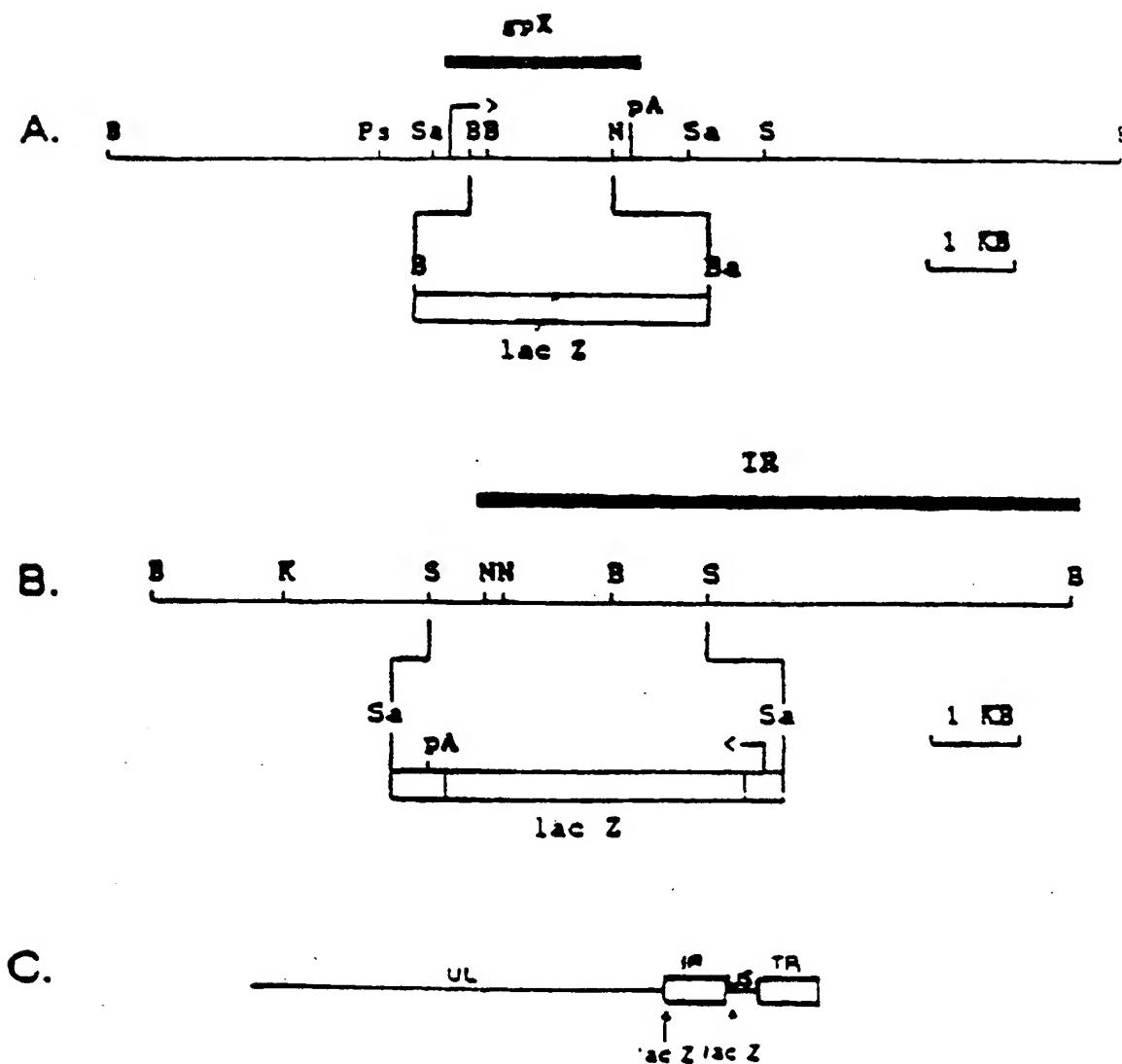


Figure 16

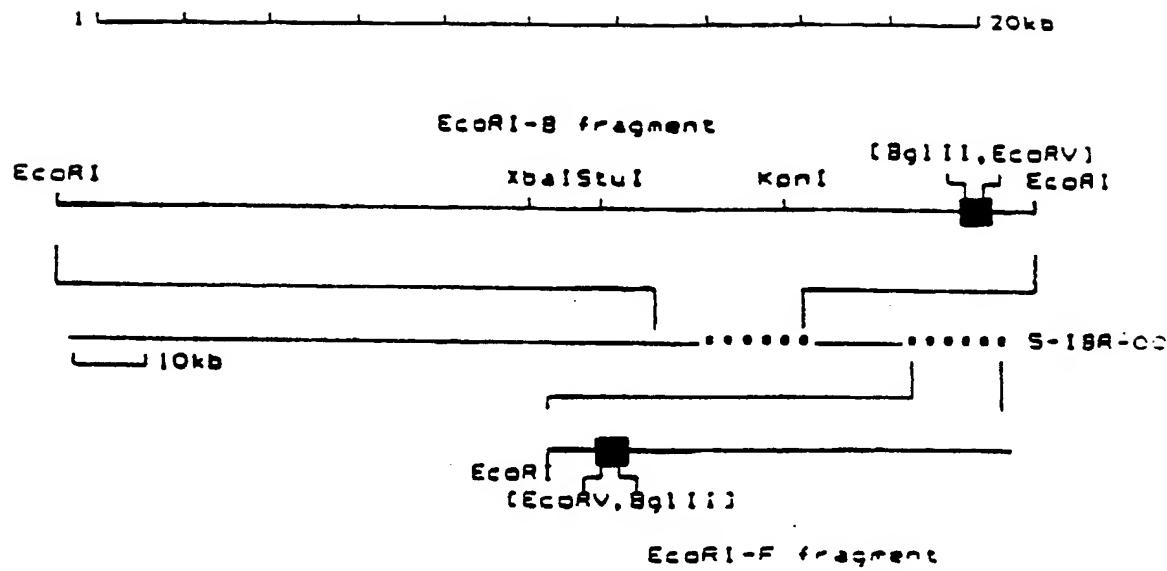


Figure 17

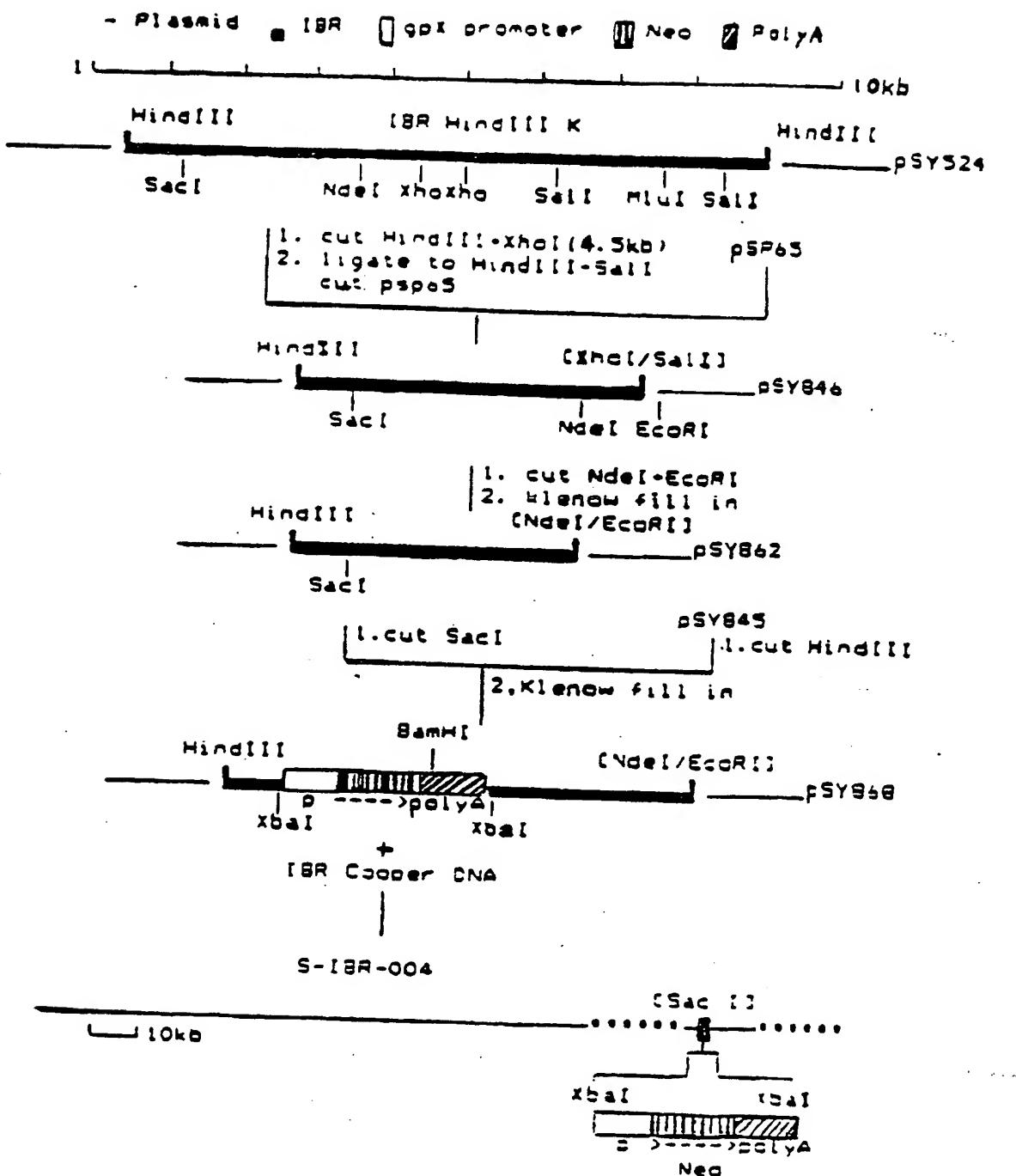


Figure 18

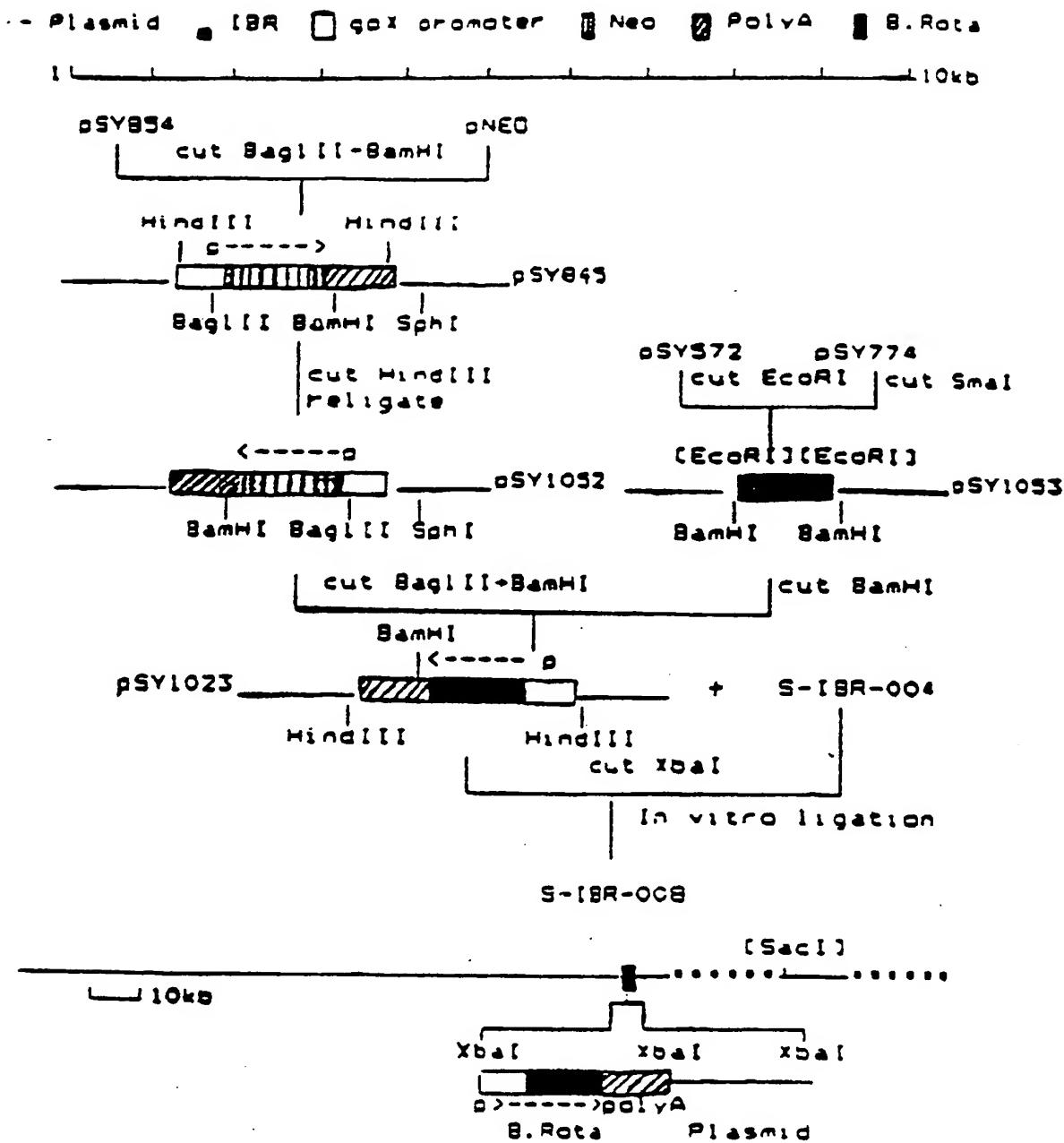


Figure 19

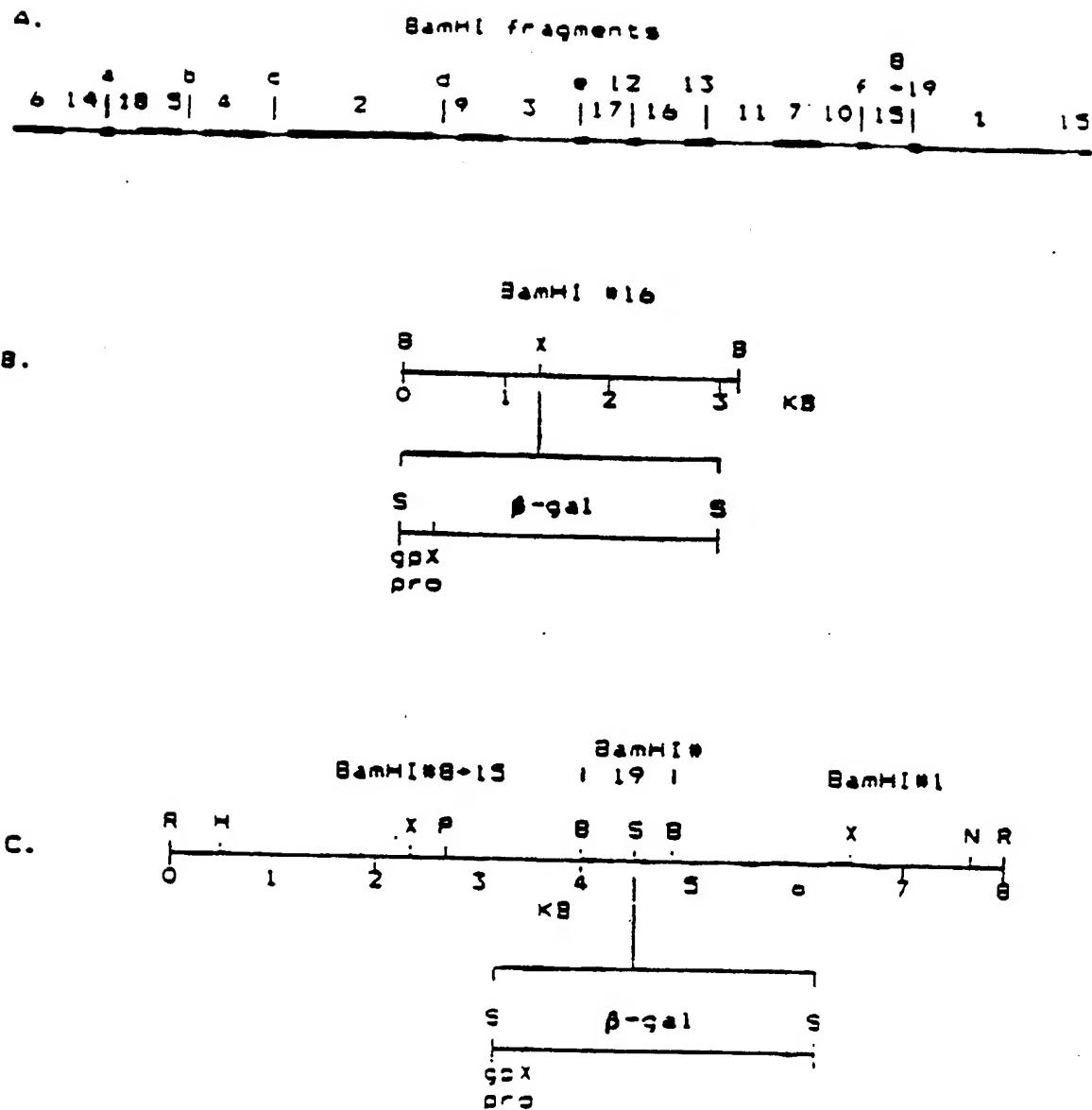


Figure 20

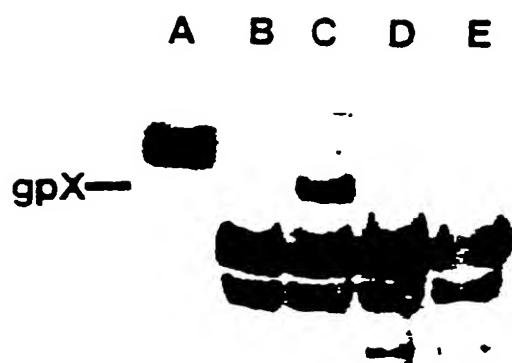
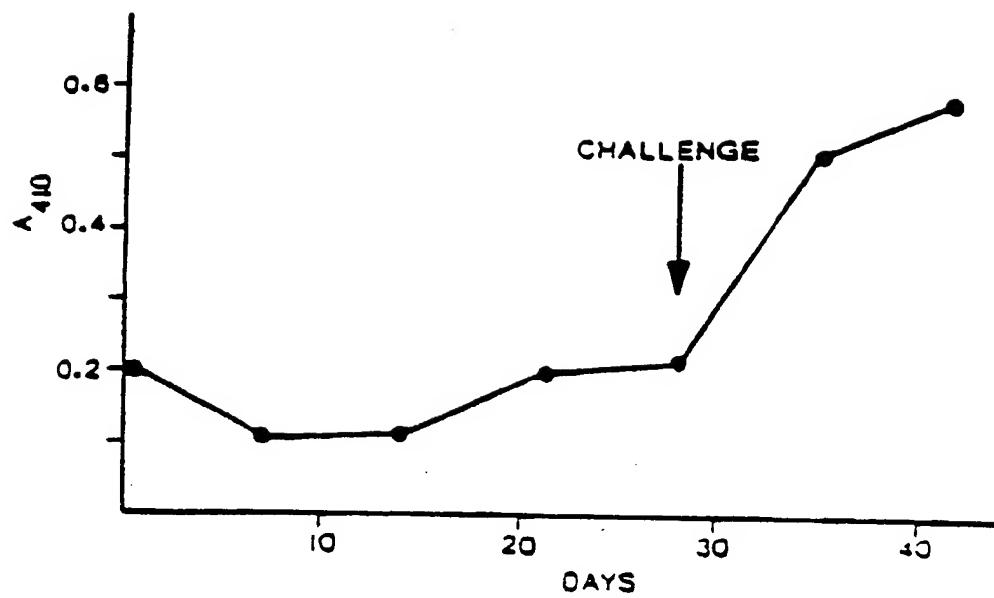


Figure 21





European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 97 10 3457

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.)						
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim							
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 81, June 1984, WASHINGTON US, pages 3365-3369, XP002034327 C.P.GIBBS ET AL.: "Extensive homology exists between Marek disease herpesvirus and its vaccine virus, herpesvirus of turkeys" * the whole document * ---	1-7	C12N15/86						
P,X	WO 86 00528 A (GENEX CORPORATION) 30 January 1986 * page 27, line 27 - page 28, line 5 * -----	1-7							
			TECHNICAL FIELDS SEARCHED (Int.Cl.)						
			C12N						
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>3 July 1997</td> <td>Cupido, M</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	THE HAGUE	3 July 1997	Cupido, M
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THE HAGUE	3 July 1997	Cupido, M							
CATEGORY OF CITED DOCUMENTS		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document							
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document									

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